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Interaction of beta-adrenergic receptors, NNK, and ethanol in selected pancreatic carcinoma cell lines

Diann Lynn Weddle

To the Graduate Council:

I am submitting herewith a dissertation written by Diann Lynn Weddle entitled "Interaction of beta-adrenergic receptors, NNK, and ethanol in selected pancreatic carcinoma cell lines." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Hildegard Schuller, Major Professor

We have read this dissertation and recommend its acceptance:

David O. Slauson, Kevin Hahn, John L. Bell

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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INTERACTION OF BETA-ADRENERGIC RECEPTORS, NNK, AND ETHANOL IN SELECTED PANCREATIC CARCINOMA CELL LINES

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Diann Lynn Weddle, DVM
December, 1999

Dedication

My Lord, Christ

My ever constant, sure, and faithful companion throughout my life

and

Max and Jean Weddle

Your encouragement, support, and devotion have been true, divine blessings

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Abstract

Pancreatic carcinoma is the 4th leading cause of cancer death in people although it ranks 11th in overall cancer incidence. The most common primary malignancy of the pancreas is ductal adenocarcinoma which represents 75% of all exocrine pancreatic neoplasms. The pathophysiology of pancreatic carcinoma remains unclear, if not controversial. Tobacco and alcohol are risk factors. Ki-ras and p53 commonly occurring genetic mutations, 75% and 50% respectively. However, the interconnection between these risk factors and genetic mutations and their role in pancreatic carcinogenesis has not been consistent. It has been reported that patients who smoke and drank had a lower incidence of Ki-ras mutations than patients who only smoked or consumed alcohol. Furthermore, it has been reported that patients without p53 or Ki-ras mutations have shorter survival times than patients who had one of the mutations.

In a transplacental hamster model, it was shown that the tobacco-specific nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) caused exocrine pancreatic adenocarcinoma in the offspring of pregnant hamsters pre-exposed to ethanol in the drinking water, where as exposure to NNK alone caused tumors of the respiratory tract. It has been demonstrated that these pancreatic tumors in the offspring of dams that received ethanol did not have p53 or Ki-ras mutations and appeared to develop tumors independent of these alterations. Tobacco is also a risk factor for various pulmonary cancers. A beta-adrenergic receptor mediated mitogenic pathway has been identified in human peripheral pulmonary

adenocarcinoma cell lines. NNK has also been shown to bind to beta-adrenergic receptors in these cells.

Using radioligand binding techniques, β_1 and β_2 -adrenergic receptor subtypes were found in membrane preparations from fetal hamster pancreas and from 4 human pancreatic adenocarcinoma cell lines. In the fetal hamster pancreas of offspring from dams who did not receive ethanol, there was a higher proportion of β_1 receptors. In the fetal pancreas of offspring from ethanol treated dams and in the human pancreatic carcinoma cell lines, a higher proportion of β_2 receptors was demonstrated. As studied with competition assays in the fetal pancreas, NNK bound to the beta-adrenergic receptors. In the ethanol treated pancreas, the competition curve shifted to the left suggesting an increased affinity of NNK to the receptors. As studied by 3^H-thymidine incorporation, increasing concentrations of NNK did not result in increased DNA synthesis. Maintenance of these cell lines in media containing ethanol did not change this observation. However, simultaneous treatment with NNK and a β_2-antagonist resulted in marked inhibition of DNA synthesis in all cell lines under both general and ethanol media maintenance conditions.

These findings did not fully support the central hypothesis that NNK would induce proliferation of human pancreatic carcinoma cell lines through beta-adrenergic receptors and that the effect would be enhanced in the presence of ethanol. These findings did demonstrate a potential role of beta-adrenergic receptors, particularly β_2, perhaps in concert with NNK, in the cell cycle regulation of these cells.

Chronic alcohol consumption is a risk factor for chronic pancreatitis which is also a risk factor for pancreatic cancer. Beta-adrenergic receptors have been shown to activate

pathways involving phospholipases which can trigger the release of arachidonic acid. Recent evidence has demonstrated that cyclooxygenase-2 expression is upregulated in human pancreatic adenocarcinomas. These findings together suggest that a link between beta-adrenergic receptors and the arachidonic acid pathway may exist and provide a pathophysiologic role in the development of this cancer type.

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PART II: VERIFICATION OF BETA-ADRENERGIC RECEPTORS AND DETERMINATION OF SUBTYPE

LIST OF ABBREVIATIONS

ATCC - American Type Culture Collection

BSA - bovine serum albumin

cAMP - cyclic adenosine monophosphate

cDNA - complementary deoxyribonucleic acid

CsCl - cesium chloride

CYP - [^{125}I]-(-)Iodocyanopindolol

dATP - deoxy-adenine triphosphate

DBN - *N*-nitrosodibutylamine

dCTP - deoxy-cytosine triphosphate

DEN - nitrosodiethylamine - model *N*-nitroso compound

dGTP - deoxy-guanosine triphosphate

DNA - deoxyribonucleic acid

dNTP - deoxy-nucleotide triphosphate

DMBA - 7,12-dimethylbenz[a]anthracene - polycyclic aromatic amine

dTTP - deoxy-thymidine triphosphate

EDTA - ethylene diamine tetratacetic acid

FBS- fetal bovine serum

GIT - guanidine isothiocyanate

Ki-ras - intracellular signalling factor

LIST OF ABBREVIATIONS

M-MLV - Maloney-Murine leukemia Virus - source of reverse transcriptase enzyme in the reverse transcription reaction

MAP - mitogen activated/associated protein

NAB - *N'*-nitrosoanabasine - tobacco-specific nitrosamine

NAT - *N'*-nitrosoanatabine - tobacco-specific nitrosamine

NNA - iso-NNAL--[4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol] - metabolite of NNK and tobacco-specific nitrosamine

NNAC (iso-NNAC) - 4-(methylnitrosamino)-4-(3-pyridyl)-1-butyric acid - metabolite of NNK and tobacco-specific nitrosamine

NNAL - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol - metabolite of NNK and tobacco-specific nitrosamine

NNK - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone - nicotine derived carcinogen

NNN - *N'*-nitrosonornicotine - nicotine derived carcinogen

p53 - tumor suppressor gene

PBS - phosphate buffered saline

RNA - ribonucleic acid

RNasin - placental RNase inhibitor

RT-PCR - reverse transcriptase polymerase chain reaction

PART I

INTRODUCTION AND OVERVIEW

Chapter 1: Epidemiology and of pancreatic carcinoma

I. Epidemiology

Pancreatic carcinoma is the 4th leading cause of cancer death in people although it ranks 11th overall in cancer incidence. Survival rates reported are as follows: 3-5%, 5 years; 9%, 2 years (Gold, 1998; Kinjo, 1998). This high mortality rate is attributed to the late diagnosis of the disease and its propensity to metastasize. The most common primary malignancy of the pancreas is ductal adenocarcinoma which represents 75% of all exocrine pancreatic neoplasms (Wilentz, 1998).

This neoplasm has been considered "mainly a disease of elderly urban dwellers" (Kinjo, 1998). Eighty percent of patients are between the ages of 60-80 (Kinjo, 1998; Gordis, 1993). However, increasingly, younger individuals and more women are developing this neoplasm. Nineteen and ninety-eight cancer statistics estimate that there will have been 29,000 new cases and 28,900 deaths associated with pancreatic cancer (Landis, 1998). Rates are higher among blacks (both men and women) than among whites. Migrant studies, primarily on immigrant Japanese, have shown increased incidence rates among immigrant Japanese in the US compared to native Japanese (Gold, 1998; Buell, 1968). In addition, rates are higher among American blacks than African blacks. There have been studies reporting that pancreatic cancer is more frequent in Jewish individuals than in catholic or Protestants.

In addition, the rates of Mormons and non-Mormons in Utah are low compared to the overall rates of the US (Gold, 1998; Lyon, 1976). These findings suggest that genetic, environmental, and dietary factors may influence the development of this disease.

Chapter 2: Tobacco and ethanol as risk factors

I. The risk factor tobacco

Exposure to tobacco products is a better risk factor. Most studies (including eight prospective studies, a Japanese cohort study, numerous case control studies) have shown an increased risk of pancreatic cancer associated with cigarette smoking (Doll, 1976; Hirayama, 1989; Shibata, 1994). In addition, hyperplastic changes in pancreatic duct cells including atypical nuclear changes have been found in smokers at autopsy (Fraumeni, 1975). These changes were also reported to increase with smoking dose.

Exposure to tobacco products is a well-established risk factor for a number of conditions including chronic lung disease, heart disease, and a variety of cancer types. Tobacco is composed of nicotine and several thousand toxicants and irritants, including multiple tobacco specific nitrosamines (Hecht, 1998). The nitrosamines have been identified as the most potent cancer causing agents in tobacco products and will be the main focus of this section. Nitrosamines are formed by nitrosation of secondary and tertiary amines (Hecht, 1998). This process can occur endogenously in the gut. In the case of the tobacco-specific nitrosamines, they form from nicotine during the curing and storage processes of the tobacco product and in the host organism. Nicotine itself is a tertiary amine with a pyrrolidine and pyridine ring. The two most abundant nitrosamine products of nicotine are NNN [N'-nitrosonornicotine] and NNK [4-(methylnitosamino)-1-(3-pyridyl)-1-1butanone]. These along with the metabolic products of NNK {NNAL--[4-(methylnitrosamino)-1-(3-

pyridyl)-1-butanol]} andNNA {iso-NNAL--[4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol] and iso-NNAC--[4-(methylnitroamino)-4-(3-pyridyl)butyric acid]} all occur in tobacco smoke and are referred to as tobacco-specific nitrosamines. Also included in this group are NAT [N'-nitrosoanatabine] and NAB [N'-nitrosoanabasine] (Hecht, 1988; Hecht, 1994).

Nicotine in and of itself has not been proven to be carcinogenic when tested in laboratory animals; however, the nitrosamines, particularly NNN and NNK are potent carcinogens (Hecht, 1998). NNK and its metabolite NNAL have been shown in multiple carcinogenesis studies to be the most active of these nitrosamines (Hecht, 1998; Schuller, 1998). NNK is a potent pulmonary carcinogen in rats, mice, and Syrian golden hamsters. In addition, tumors of the nasal cavity, liver, trachea, and pancreas have been induced in these species. There is substantial evidence that supports the idea that nitrosamines are principal components in the development of cancers of the lung, oral cavity, esophagus, and pancreas. Reactive metabolites formed from NNK induce the formation of O^6-methylguanine DNA adducts, implicated in the activation of Ki-ras protooncogenes (Belinsky, 1989; Hecht, 1993).

II. The risk factor ethanol

There is epidemiological data strongly linking alcohol consumption as a risk factor for cancer. These include studies showing national per capita alcohol consumption paralleling age-adjusted mortality from cancer, studies showing groups of people who consume large amounts of alcohol have increased cancer risk, studies showing groups of people who traditionally abstain from alcohol (Mormons, Seventh-Day Adventists) have decreased

cancer risk, and studies showing patients with malignancies have an associated history of alcohol consumption (Jensen, 1979; Lemon, 1964; Lyon, 1977; Lyon, 1976; Monson, 1975; Mufti, 1992; Tuyns, 1970; Vincent, 1963).

However, multivariate analysis of epidemiological studies to try to determine a direct effect of ethanol on cancer development has produced inconsistent results. Included in these data are those regarding alcohol consumption and pancreatic carcinoma. Alcohol is known to cause chronic pancreatitis and chronic pancreatitis is thought to be a risk factor for pancreatic carcinoma. However, the causative link between the two is still unclear (Gold, 1998).

In regard to the effects of ethanol on cancer, when looking at animal studies or carcinogenesis studies the reasons for the inconsistences become more apparent. In an extensive review by S. I. Mufti (1992), it was determined that studies in which ethanol was administered to animals through the life span of the animal used failed to show clear results that would indicate ethanol as carcinogenic in and of itself. Mufti further stated that the argument could be made that perhaps alcohol could be weak in its carcinogenic effect and take years to establish. Most studies addressing ethanol's link to carcinogenesis have involved induction of the carcinogenesis by known carcinogens. Mufti reports that the results of these studies have depended on a number of variables: species used, carcinogens used, dose/schedule of ethanol administration, and its relation to timing of carcinogen administration.

Ethanol may play a role in either modifying the initiation of carcinogenesis or act as a tumor promoter depending on when administered. It has been proposed that the

inconsistent results noted in multivariate analytical epidemiological applications and in animal studies may have been caused by the failure to distinguish between these two exposure situations.

Several proposed potential pathways for ethanol to influence the initiation process include increasing intracellular carcinogen concentrations (Arimoto, 1982), increasing cell membrane permeability (Smith, 1971), and/or increasing carcinogen metabolism (Kalant, 1976; Liu, 1975; Mezey, 1976; Rubin, 1970; Rubin, 1968). Ethanol is a known inducer of cytochrome p450 enzymes (including p450IIE1 which metabolize volatile low molecular weight nitrosamines) and pretreatment with ethanol is known to enhance metabolism of drugs and carcinogens that use this system (Anderson, 1992). These mechanisms could result in greater availability of a carcinogen to exert its actions. Discrepancies have arisen in cases where nitrosamines have been used to induce cancer and the nitrosamines were co-administered with ethanol. In these situations, some nitrosamines has been shown to increase extra-hepatic tumor numbers while reducing the number of liver tumors. As an example, C57BL/6 mice given repeated oral doses of *n*-nitrosodimethylamine (NDMA) in 40% ethanol experienced a 50% reduction in liver tumors. However, these mice developed nasal tumors which were not seen in animals receiving only NDMA (Griciute, 1981). The reason for this apparent discrepancy is that ethanol can act as a competitive inhibitor of the enzyme demethylase which is involved in the metabolism of nitrosamines in the liver (Miller, 1984; Anderson, 1992). Finally, in addition to the above stated pathways for initiation, ethanol is cytotoxic. Cytotoxic injury can result in cell death and secondary hyperplasia (Mufti, 1992). This effect has been linked to the potential effects of ethanol

inducing precursor neoplastic lesions associated with chronic inflammatory conditions of the liver (Lieber, 1983; Mufti, 1992).

A number of studies indicate that ethanol can act as a tumor promoter and that it meets several criteria of tumor promoters: not carcinogenic itself alone, multiple chronic exposures needed for effect, and acts above a threshold dose (Mufti, 1992). Multiple molecular mechanisms have been proposed to explain ethanol's potential promoter effects.

First, ethanol consumption can be conducive to lipid peroxidation through free radical formation (Dianzani, 1985). This is thought to occur as a result of excessive reducing equivalents and the generation of reactive oxygen species following the induction of the microsomal ethanol oxidizing system by the ethanol. Increased levels of lipid peroxidation products (ethane, hepatic diene conjugates) have been noted in studies of rats consuming ethanol on a chronic basis (Szebeni, 1986; Mufti, 1991; Mufti, 1992). This increase could be inhibited by supplementation with vitamin E (Mufti, 1992).

Second, ethanol may alter the efficiency of DNA repair enzyme systems (Mufti, 1992). Aberrant methylation of hepatic DNA has been observed with long term ethanol consumption (Barrows, 1981; Mufti, 1988). In addition, chromosomal alterations have been noted in chronic alcohol users (Mufti, 1992; Alvarez, 1983). A logical consequence of the induction of DNA strand breaks by free radicals along with decreased efficiency of DNA repair would be a significant contribution to tumor promotion or induction. Pretreatment of rats with ethanol before treatment with carcinogens N-nitrosomethylamine and N-nitrosomethylbenzylamine caused increased levels of O^6-methylguanine, a known DNA adduct formed by nitrosamines (Garcea, 1984) which causes activating point mutations in

the Ki-ras gene (Belinsky, 1989).

Third, alcohol may contribute to immune suppression. In alcoholic people, lymphocytes have decreased intracellular cells of cAMP (Diamond, 1987; Nagy, 1988) and impaired mitogenic activity (Sorrell, 1972). Decreased T-cell number and impaired function along with impaired phagocytic activity has been observed in in vivo and in vitro studies. Fourth, the cytotoxic effects of ethanol could also cause tumor promotion. A number of tumor promoters cause a proliferative response that involve primarily expansion of clonal populations of initiated cells (events that could also establish appropriate conditions for precursor lesions chronic inflammatory liver conditions).

Chapter 3: Statement of the problem-controversies in pancreatic carcinogenesis

I. Controversy over risk factors

At present, there is controversy over the risk factors involved in the development of pancreatic carcinoma. Tobacco is a known risk factor (Doll, 1976; Hirayama, 1989; Shibata, 1994). Alcohol consumption is also thought to be a risk factor. Alcohol is known to cause chronic pancreatitis and chronic pancreatitis is thought to be a risk factor for pancreatic carcinoma. However, the causative link between the two is still unclear. In addition, multivariate analysis of epidemiological studies to try to determine a direct effect of ethanol on development of pancreatic carcinoma has produced inconsistent results (Gold, 1998; Gordis, 1993).

II. Controversy of role of genetic mutations

In addition to the apparent controversy regarding risk factors, there are apparent discrepancies in the role of specific genetic mutations in the development of this cancer type. Point mutations in the Ki-ras gene are frequent (75%) and occur as an early event in human pancreatic adenocarcinoma (Almoguera, 1988; Hruban, 1993; Pellegata, 1994; Rozenblum, 1997; Terhune, 1998). In addition, 50% of human pancreatic adenocarcinomas have p53 mutations (Pellegata, 1994; Rozenblum, 1997) Malats, et al (1997) reports that human patients who smoke and drank had a lower incidence of Ki-ras mutations in their pancreatic adenocarcinomas than those who only smoked or consumed alcohol. These findings suggest

an interaction between alcohol and tobacco. It has also been reported that patients with pancreatic adenocarcinomas which lacked both p53 and Ki-ras mutations have shorter survival times than patients with one of the mutations (Dergham, 1997).

In a transplacental hamster model, it was shown that the tobacco-specific nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) caused exocrine pancreatic cancer in the offspring of pregnant hamsters who were previously exposed to ethanol in the drinking water (Schuller, 1993), where as exposure to NNK alone caused tumors of the respiratory tract. In the offspring of dams that received ethanol and NNK, there were significant incidences of pancreatitis, ductal and acinar hyperplasia, pancreatic adenocarcinomas, and pheochromocytomas. These findings were more significant in female offspring where the incidences of hyperplastic lesions and pancreatic adenocarcinomas were 77% and 59%, respectively (Schuller, 1993). In addition, according to preliminary, unpublished data from the laboratory of a co-investigator, pancreatic tumors in the offspring of dams that received ethanol did not have p53 or Ki-ras mutations and appeared to develop tumors independent of these alterations.

III. Potential controversy in the role of ethanol

In chapter 2, section II of part I of the dissertation, there are a number of mechanisms by which ethanol can influence carcinogenesis. Ethanol can induce cytochrome p450 IIE1 enzymes which are responsible for the metabolism of nitrosamines (Anderson, 1992). This would potentially result in increased levels and increased availability of carcinogenic metabolites. NNAL is a potent carcinogen and is the primary metabolite of NNK in the

hamster (Jorquera, 1992). An increased rate of metabolism in the lungs and livers of fetal hamsters exposed in utero to ethanol has been observed (Jorquera, 1992). However, preliminary, unpublished data, from the laboratory of a co-investigator does not show significant differences in levels of NNAL in the pancreases of fetal hamsters exposed in utero to ethanol compared to those without exposure.

Ethanol may alter the efficiency of DNA repair enzymes (Mufti, 1992). Aberrant methylation has been observed with long term ethanol consumption (Barrows, 1981; Mufti, 1988), and chromosomal alterations have been noted in chronic alcohol users (Mufti, 1992; Alvarez, 1983). In addition, pretreatment of rats with ethanol before treatment with carcinogens *N*-nitrosomethylamine and *N*-nitrosomethylbenzylamine caused increased levels of O6-methylguanine, a known DNA adduct formed by nitrosamines (Garcea, 1984). This adduct causes point mutations in the Ki-ras gene (Belinsky, 1989). As stated earlier in chapter 3, section II of part I, point mutations in Ki-ras are frequent and occur as an early event in human pancreatic adenocarcinoma (Almoguera, 1988; Hruban, 1993; Pellegata, 1994; Rozenblum, 1997; Terhune, 1998), and in addition, 50% of human pancreatic adenocarcinomas have p53 mutations (Pellegata, 1994; Rozenblum, 1997) Also stated in this same section, unpublished data from the laboratory of a co-investigator, pancreatic tumors in the offspring of dams that received ethanol did not have these mutations and appeared to develop tumors independent of these alterations. In addition, epidemiologic data (Malats, 1997) reports that human patients who smoke and drank had a lower incidence of

Ki-ras mutations in their pancreatic adenocarcinomas than those who only smoked or consumed alcohol. These findings suggest that there is an interaction between tobacco and ethanol. However, the specifics of this interaction remain unknown.

Chapter 4: Potential mechanisms of carcinogenesis as relates to NNK and beta-adrenergic receptors

I. The role of NNK

Tobacco is an undisputed risk factor for pancreatic carcinoma (Doll, 1976; Gold, 1998; Hirayama, 1989; Shibata, 1994). NNK is one of the principal carcinogens in tobacco (Hecht, 1998). The primary focus on the carcinogenetic action of NNK has revolved around its ability to induce the formation of O^6-methylguanine DNA adducts. These adducts are implicated in the activation of Ki-ras protooncogenes (Belinsky, 1989). There is evidence emerging in this laboratory that NNK can activate MAP kinases directly (Jull, 1999). This data has been derived from experiments using normal fetal pulmonary neuroendocrine cells which presumably do not have Ki-ras mutations or other mutations. Therefore, suggesting that NNK could induce positive proliferative effect by direct activation of signal transduction pathways via stimulation of a mitogen without having induced a mutation. NNK has also been shown to induce proliferation of fetal lung cells as measured by increased cell counts compared to controls.

II. The potential role of the beta-adrenergic system

The autonomic nervous system is widely distributed throughout the body and functions in conjunction with the endocrine system to provide involuntary regulation for optimum internal homeostasis and fine control for many parameters involved in cell behavior

(Lefkowitz, 1990). Given these factors it is surprising that little attention has been given to the potential role of this system in the development of cancer. There is emerging evidence that the sympathetic branch of the autonomic nervous system could play a contributing role if not initiatory role in the development of some environmentally related cancers (Merryman, 1997; Park, 1995; Schuller, 1998; Schuller, 1997; Schuller, 1995).

For the past 20 years, there has been an increase in the incidence of peripheral adenocarcinoma in industrialized countries (Wynder, 1994). These neoplasms exhibit a papillary pattern; the cell composition is primarily of cells with Clara cell features with a small proportion of cells with alveolar type 2 features (Linnoila, 1991). This increase has been observed not just in smokers but nonsmokers with no apparent justification (Wynder, 1994). In addition, there is a parallel increase in chronic respiratory disease. Chronic lung disease and bronchitis are known risk factors for the development of cancer (Devalia, 1994). Patients with these conditions are often on intermittent therapy consisting of beta-adrenergic agonists for many years. It has been well established that NNK administered to hamsters under hypoxic conditions induces neuroendocrine tumors via a pathway that involves nicotine receptors (Schuller, 1995). It has also been established that hamsters exposed to ambient air develop non-neuroendocrine tumors of Clara cell origin (Schuller, 1990). Because of the structural similarities of NNK with β-adrenergic agonists, the question logically arose as to what role could chronic beta-adrenergic stimulation play in lung carcinogenesis.

Using human pulmonary adenocarcinoma cell lines derived from peripheral lung adenocarcinomas (NCI-H322 and NCI-H441), a beta-adrenergic receptor mediated growth

pathway was demonstrated (Park, 1995) In cell proliferation assays, significant proliferation was seen in these cell lines when exposed to beta-adrenergic agonist, epinephrine and isoproterenol. This proliferation was inhibited by a beta-adrenergic antagonist, but not by an alpha-adrenergic antagonist. In addition, the cell proliferation could also be inhibited by an inhibitor of adenylate cyclase (SQ22536) and inhibitor of cAMP intracellular formation (carbachol). Furthermore, cAMP assays supported these findings by showing activation of this enzyme system by epinephrine, isoproterenol and forskolin in these cell lines. These experiments were also performed in a small cell lung cancer cell line (NCI-H69) which did not show the positive proliferative responses. Finally, radioligand binding studies demonstrated expression of beta-adrenergic receptors in the adenocarcinoma cells.

As stated earlier, relatively little attention has been given to potential role β-adrenergic receptors may have in a functional proliferative response. As pointed out in a review of cAMP mediated cell proliferation by Dumont, et al (1989). Cyclic AMP's role in inhibition of mitogenesis has been regarded has been well established and perhaps overstated. The review lists a fair number of cell systems in which the mitogenic role of cAMP has been documented. In several of these cell systems, the initiation of the cAMP response is thought to occur via β-adrenergic receptors. There the above experiments appear to be the first evidence of a mitogenic pathway involving a β-adrenergic receptor in pulmonary carcinogenesis. The introductory segment of this chapter will later include a discussion on the structure of NNK, a well-documented carcinogen, and its similarity to β-agonists.

III. Effects of ethanol on beta-adrenergic receptors

There are multiple studies showing ethanol to have an effect on either density or the responsiveness or both of β-adrenergic receptors. In one study, ethanol was provided to rats over a 3 month period. The hearts of these rats were found to have a significant reduction in receptor number, decreased levels of cAMP, and significantly higher levels of norepinephrine and epinephrine compared to controls. The affinity of the receptors was unchanged. The decreased density and adenylate cyclase activity (as measured by cAMP levels) were attributed to increased levels of the catecholamines and resultant compensatory down regulation (Koga, 1993). Another in vitro study using ventricular membranes from rats given ethanol orally for a 3 week period found a decrease in the number of β-adrenergic receptors compared to untreated controls (Pohorecky, 1992).

These results are not unlike studies on the levels of β-adrenergic receptors in lymphocytes of chronic alcoholics. Again, compensatory down regulation secondary to increased endogenous catecholamine levels was sited as probable cause of the results (Maki, 1990). However, this same study noted a rapid reversal in receptor number and functioning following abrupt ethanol withdrawal. Similar findings with regard to this latter finding was noted by Banerjee, et al (1978).

In an in vitro study using myocardial membranes taken from rabbits which had not been treated with ethanol, basal cAMP levels were not affected by ethanol concentrations less than 425 mM. Higher concentrations of ethanol decreased cAMP levels. However, ethanol in the presence of isoproterenol and Gpp (NH)p (an activator of the Gs regulatory protein) increased the cAMP levels. Other studies examining the effects of ethanol on

adenylate cyclase activity in lymphoma cells (Rabin, 1983) and neural cortex (Saito, 1985) found increases in basal adenylate cyclase activity with ethanol.

Prenatal alcohol exposure altered the β_1-adrenergic receptor binding in brown adipose tissue in postnatal rats (Zimmerberg, 1995). On day 5, there was a significant increase in receptor number compared to controls. Although, the receptor numbers declined from day 5 to day 20 in both treated and untreated control groups, receptor numbers in the ethanol treated group remained significantly higher than controls. However, in this prenatal exposure study, the levels of norepinephrine were higher than that of controls, suggesting the expected compensatory down regulation of receptors may be altered in this model.

Chapter 5: Project overview: hypotheses and objectives

I. The central hypothesis

The focus of this research dissertation will be to focus on one aspect of the carcinogenetic potential and interaction of NNK, ethanol, and beta-adrenergic receptors in pancreatic carcinoma. The central hypothesis is that NNK will induce proliferation of pancreatic carcinoma cell lines through beta-adrenergic receptors and that this effect will be enhanced in the presence of ethanol.

II. The specific hypotheses and objectives

1. Ethanol treatment via the drinking water in pregnant hamsters will increase the receptor density of beta-adrenergic receptors in fetal pancreas. In addition, pancreatic carcinoma cell lines contain beta-adrenergic receptors. These processes will be examined by radioligand binding studies, saturation and competition assays, using a radioligand with a high affinity to beta-adrenergic receptors and nonselectivity to subtypes β_1 or β_2. The objectives will be to determine if beta-adrenergic receptors are present, which subtypes are present, and at what proportion. Saturation binding studies will be used to determine receptor number and affinity in the fetal pancreas. Competition binding studies will be used to determine which subtypes are present and relative proportion of those subtypes in the fetal pancreas and in four pancreatic carcinoma cell lines. Further verification of binding results will be completed by identification of specific cDNA sequence expression for beta-adrenergic

receptor subtypes using nonquantitative reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR products will be sequenced and compared to published sequences for beta-adrenergic subtypes.

2. NNK will induce proliferation of pancreatic carcinoma cell lines. Ethanol treatment of these cell lines enhances this effect. The proliferation will be blocked by beta-adrenergic antagonists. The objectives will be to demonstrate a positive proliferative effect with treatment of NNK compared to controls which received no treatment and to demonstrate the demonstrate the influence of beta-adrenergic receptors in this process by demonstrating blockade of this proliferation via beta-adrenergic antagonists. Furthermore, subtype selective antagonists will be used to determine if this influence was subtype specific. In addition, further objectives will be to examine differences in those results after the pancreatic cell lines had been exposed to ethanol. These processes will be studied by measuring DNA synthesis as a function of tritiated thymidine incorporation in four different pancreatic carcinoma cell lines. The cells will be exposed to increasing concentrations of NNK, no NNK, or subtype selective antagonists with NNK under two media environmental conditions. The two conditions will be cells having been maintained in general media conditions and cells having been maintained in media containing ethanol. Pancreatic carcinoma cell lines are chosen because at present there is no good in vitro system for studying normal human pancreatic duct cells.

Chapter 6: The hamster transplacental model of carcinogenesis

The fetal tissues used in the radioligand binding studies in part 2 of this dissertation were derived from a hamster transplacental model of carcinogenesis. The author of this dissertation wishes to present a brief overview of this model.

I. History

With regard to the history of transplacental carcinogenesis, the first experiments in this area have been attributed to researchers by the name of Shay and Symeonidis (early 1950's). There assumption was that certain agents may result in tumor development in adults even though exposure only occurred during a limited time frame in utero. (Stavrou, 1984) Before this however, there was evidence published in 1928 by Shabad that showed an increased incidence of lung tumors in young mice of dams who had been treated with tars. (Ivankovic, 1984) Then in 1970, Herbst and his group found a direct correlation between the presence of vaginal carcinoma in young girls and diethylstilbestrol therapy in their mothers (Herbst, 1971). There has also been reports of the sons of these mothers developing testicular tumors (Stavrou, 1984).

II. Anatomic and physiologic considerations relevant to the animal model

There are some basic anatomic and physiologic factors that have to be considered when using transplacental models of carcinogenesis (Schuller, 1984). They include length of pregnancy of a given species, placentation type, differences in metabolic ability of the

placenta and target fetal organs, and level of tissue/organ differentiation. The length of pregnancy of a given species can affect the time interval of susceptibility to a certain agent. The number of layers separating the maternal and fetal blood (placentation type) may affect the passage ability of a given agent. Metabolic ability of target organs is important because some agents require metabolic activation to cause their effect. Finally, the level of tissue/organ differentiation reflects the cells metabolic ability/capacity and hence their ability to metabolize a given agent as well as the expression levels of cell surface receptors important in the initiation of mitogenic signal transduction pathways.

Most carcinogens act as transplacental carcinogens during the third trimester (Schuller, 1984). This may be a reflection of the following points:

-agents that affect early in pregnancy affect a wide variety of developmental processes that will more likely have widespread effects and result in teratogenesis or death.

-a more developed organ/tissue is needed because metabolism of the agent may be required and cell surface receptors need to be expressed.

III. The hamster model

Transplacental studies have provided much information on the mechanisms of carcinogenesis. Much of what is known about the mechanisms of transplacental carcinogenesis of nitrosamines has been derived from studies using the Syrian golden hamster (Schuller). In general, the primary target of these compounds when given to gestating hamsters is the respiratory tract of the F1 offspring. The susceptibility of the fetal tissues to these carcinogens is generally higher in the third trimester (Schuller, 1984).

In studies using nitrosodiethylamine (DEN), a model N-nitroso compound, a single dose of the compound given to pregnant hamsters on one of the first 11 days of gestation did not result in the development of respiratory tumors. Respiratory neoplasms were not seen until the dose was given on one of the last 4 days of gestation (Schuller, 1984).

In other transplacental studies, a single oral dose of 7,12-dimethylbenz[a]anthracene (DMBA), a polycyclic aromatic amine, given to hamsters on days 8, 9, or 15 of pregnancy resulted in a high incidence of a variety of tumors in the offspring, including skin, kidney, ovaries, thyroid glands, and central nervous system (Rustia, 1977). Tumors of the respiratory tract have been induced in the offspring of female hamsters having received a variety of nitrosamines during pregnancy. *N*-nitrosodibutylamine (DBN) given as a single subcutaneous injection to dams on one of the last 4 days of pregnancy resulted in polyps on the larynx, trachea, in the main stem bronchi, and adenocarcinomas of the nasal epithelium (Schuller, 1984). NNK given as a single subcutaneous injection on day 15 or as multiple injections on days 13, 14, 15 resulted in tumor formation in 70% of the offspring within 1 year after birth (Correa, 1990). Anatomic sites included various organs including the respiratory tract. Intratracheal instillation of hamsters on day 15 of pregnancy induces tumors of the nasal cavity in 28.6% to 50% of the offspring (Schuller, 1994).

In the transplacental model used in this laboratory, NNK is instilled intratracheally on day 15 of gestation and ethanol is provided in the drinking water from day 10-15 of pregnancy. In studies measuring radioactivity of maternal, placental, and fetal tissues

following intratracheal instillation of tritiated NNK, it was found that NNK readily crosses the placental barrier quickly reaches amniotic fluid and fetal tissues and is subsequently eliminated slowly from these tissues (Jorquera, 1992).

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PART II

VERIFICATION OF BETA-ADRENERGIC RECEPTORS

AND

DETERMINATION OF SUBTYPE

Chapter 1: Introduction

I. Brief overview

This chapter is devoted to the identification and determination of sub-types of beta-adrenergic receptors in the fetal hamster pancreatic tissues and the human pancreatic carcinoma cell lines (AsPC, Panc-1, BxPC-3, and Capan-1) by radioligand binding studies. Confirmation of binding studies was achieved by reverse-transcription of mRNA specific for β_1 and β_2 genes and subsequent amplification of the generated cDNA by polymerase chain reaction.

II. The beta-adrenergic receptor

Beta-adrenergic receptors are part of the sympathetic division of the autonomic nervous system. Beta-adrenergic receptors are divided into 3 subtypes (β_1, β_2, β_3) based on pharmacologic binding preferences (Lefkowitz, 1990). Norepinephrine and epinephrine are the catecholamines that serve the peripheral β-receptors. Norepinephrine and epinephrine will bind at relatively equally to both β_1 and β_2 subtypes. Epinephrine is 10-50 fold more potent at β_2 sites (Hoffman, 1990). The gene that encodes the third subtype (β_3) was isolated by Emorine, et al (1989). This subtype is 10 fold more sensitive to norepinephrine and is resistant to antagonists such as propranolol. It is felt that this subtype may mediate responses to catecholamines at tissue sites such as adipose tissue with atypical pharmacological characteristics.

The basic structure of a β-adrenergic receptor is that of an integral membrane glycoprotein with seven transmembrane spanning domains, a series of interconnecting loops, glycosylated extracellular amino terminus, and a cytoplasmic carboxyl terminus (figure 1, appendix)(Stryer, 1995; Dohlman, 1987). They are G protein coupled receptors. Upon binding by an agonist, β-adrenergic receptors through this G protein stimulate adenlylate cyclase. Cyclic AMP is accumulated and activates cAMP-dependent protein kinase which in turn phosphorylates other proteins to exert a change in cell behavior (Lefkowitz, 1990).

III. Structure of NNK

NNK [4-methylnitrosamino)-1-(3-pyridyl)-1-butanone] is one of the most common, along with NNN and NAT, of the nitrosamines in tobacco products. Nicotine in tobacco products nitrosates to form these products which are then present in the unburned tobacco product and in smoke. The structure of NNK (figure 2, appendix) consists of a heterocyclic ring with an aliphatic side chain of 4 carbons and a terminal nitrogen (Hecht, 1988). Neurotransmitters for adrenergic receptors include epinephrine and norepinephrine. Their structures consist of a catechol ring (benzene ring with a hydroxyl group at positions 3 and 4) and a side chain with an α and β carbon and a terminal nitrogen (Hoffman, 1990; Ruffolo, 1994). An intact catechol ring is required only for α-adrenergic agonists and not β-adrenergic agonists. Increasing the steric bulk of the terminal nitrogen atom in the aliphatic side chain increases β-adrenergic specificity (Ruffollo, 1994). The heterocyclic ring and aliphatic side chain with its bulky nitrogen atom make NNK a good candidate as a β-adrenergic agonist.

IV. Beta-adrenergic receptors and the pancreas

Adenocarcinomas are neoplasms of organs with secretory function. The pancreas is no exception. Neurons of cell bodies in the celiac ganglia send postganglionic adrenergic innervation to intrapancreatic ganglia, ducts, blood vessels, and islets. Histologically, the most prevalent of the exocrine pancreatic adenocarcinomas are ductal or have mixed ductal/acinar components. The duct cell is thought to be the cell of origin of these carcinomas. (Wilentz, 1998). The pancreatic duct cell and its architectural arrangement into intercalated, intralobular, and interlobular ducts function to 1) provide a framework for the acinar tissue, 2) transport acinar secretions to the duodenum, and 3) secrete electrolytes, primarily in the form of bicarbonate. The bicarbonate rich secretory fluid secreted by the duct cell flushes the acinar secretions into the duodenum. This bicarbonate fluid also serves to neutralize the acidic secretions flowing into the duodenum from the stomach.

The role of β-adrenergic receptors in regulation of pancreatic secretion is unclear. Many authors agree that the results of experiments involving exogenerous administration of adrenergic agonists or antagonists to better understand the role of this system in the control of pancreatic secretion have been confusing (Lingard, 1983; Vaysse, 1977). One author had this to say, "Overall, the results of experiments on the effects of administration of α- and β-receptor agonists are not helpful in understanding the possible role of intra-pancreatic adrenergic nerves in regulation of pancreatic secretion.". Bicarbonate secretion is primarily controlled by secretin through secretin receptors via a cAMP dependent pathway. In vivo data can be difficult to interpret given the presence of a milieu of potential hormonal and autonomic agents acting within a dynamic system. Experiments have been conducted in both

conscienous and anesthetized animals. Results of studies are often species specific. Rats tend to have an increase in secretion secondary to β-adrenergic agents (Lingard, 1983; Furuta, 1978), where as canines tend to have a decrease (Vaysse, 1977). There tend to be differences based on whether a species is a continous feeder (rats) versus a non-continous feeder (canine) (Lingard, 1983). Continuous feeders tend to have a higher basal rate of secretion. In in vivo studies, researchers have noted that changes in hemodynamics due to the effects of β-adrenergic agonists may in part be responsible for decreases noted in bicarbonate secretion (Vaysse, 1977). Anesthesia itself can affect secretion levels in the rat.

In vitro data are available. The results are still often species specific with increases in rats and decreases in canines. An in vitro study utilizing isolated perfused rat pancreas to investigate the role of β-adrenergic receptors in the genesis of pancreatic secretion (Lingard and Young, 1983) compared the effect of isoproterenol with that of secretin. The authors found that isoproterenol stimulated the flow of a bicarbonate rich fluid. Although, the flow rate induced by isoproterenol was 70% of the maximum rate evoked by secretin, the response by isoproterenol was found to be qualitatively similar to that of secretin. Isoproterenol also stimulated the secretion of protein. This effect paralleled the secretion rate of bicarbonate. The study addressed if β-adrenergic receptor stimulation had an effect on basal secretion by evaluating that parameter in the presence of a nonselective β-adrenergic antagonist, propranolol. Propranolol did inhibit the affects of isoproterenol. These positive findings of β-adrenergic stimulation of bicarbonate secretion were typical of studies in rats (Furuta, 1978).

From the literature, it appears that few studies have been done in humans and were

in vivo studies, sometimes on patients being studied for a pre-existing condition such as peptic ulcer disease. From the few studies, β-adrenergic receptors have been found to have no apparent affect on pancreatic secretion (Raptis, 1973) or decreased secretion (Ruddick, 1973).

However, in a study using pancreatic carcinoma cell lines (BxPC-3, Hs 766T, Capan-1 and 2, Panc-1) to determine which duct cell receptors were functional, these cell lines were found to have functional beta-adrenergic receptors coupled to adenylate cyclase as measured by cAMP levels generated in response to epinephrine and isoprenaline (isoproterenol, a nonselective β agonist) (Al-Nakkash, 1996). The BxPC-3 line had the greatest affect, followed by the Capan-1 line , then Hs 766T, and the Panc-1 line. None of these cell lines responded to stimulation by secretin which lead the authors to speculate that perhaps neoplastic transformation resulted in a defect of these receptors or downregulation of their expression.

Chapter 2: Materials and methods

The objectives were to determine the presence and relative proportion of β1 and β2 subtypes of beta-adrenergic receptors. These procedures were completed using fetal pancreas derived from fetuses of pregnant hamsters and 4 different pancreatic carcinoma cell lines. The objectives were addressed using radioligand binding studies and RT-PCR. Radioligand binding studies in the form of saturation and competition studies used membrane preparations derived from the fetal tissue and the pancreatic carcinoma cells. Saturation binding studies were completed only on fetal hamster pancreas. RT-PCR was completed using RNA derived from the fetal tissue and the pancreatic carcinoma cell lines.

I. Transplacental hamster model

Syrian golden hamsters were obtained (weaned) from Charles River at 6 weeks of age and breed at 12 weeks of age. Pregnant females were randomly assigned to one of two groups. One group received water with no additives. The other group received ethanol (10% v/v, 200 proof) in the drinking water beginning on day 5 of pregnancy until day 15. On day 15, of a 16 day gestation period, the dams were anesthetized via an intramuscular injection of ketamine/xylazine. Fetuses were taken and sacrificed to obtain pancreatic tissues.

Dissected pancreatic tissue was placed in 1 ml cryogenic tubes, snap frozen in liquid nitrogen, and stored in a freezer (Cryo-Fridge; Baxter Scientific Products) at -80°C.

II. Pancreatic cell lines

Human pancreatic carcinoma cell lines (Panc-1, AsPC, BxPC-3, Capan-1) were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 5 % CO_2 in T-75 ml culture flasks in media recommended by ATCC. All cell lines are adherent cells. Panc-1 cells were maintained in DMEM (4.5 g glucose per liter) with 10% FBS. AsPC cells were maintained in RPMI 1640 (without glutamine) with 20% FBS. BxPC-3 cells were maintained in RPMI 1640 (without glutamine) with 10% FBS. Capan-1 cells were maintained in RPMI 1640 (without glutamine) with 15% FBS. Basic media (DMEM and RPMI 1640) was purchased (Biofluids; Rockville, MD); FBS (Biofluids), L-glutamine (Biofluids), and penicillin/streptomycin (were purchased and added separately. Penicillin/streptomycin were added at a concentration of 50,000 units/50,000 mg per 500 ml of media. Glutamine was added at a concentration of 5 ml of 200 mM solution for a final concentration of 2 mM.

Cells were passed when 90% confluent. When passing, media was suctioned from flask. Cells were washed with 6 ml of PBS. PBS was suctioned from the flask. One ml of trypsin was added and the flask was gentlely swirled to allow coating of the cells with the trypsin. Flask was placed on a hot plate at 37°C until cells were fully loosened. Five ml of culture media was then added to the flask. The media was pipetted vigorously to wash cells

from flask wall, thoroughly mix the cells, and break up cell clumps. One to 1.5 ml of cell suspension was then added to new T-75 ml culture flask.

III. Membrane preparation

Pooled tissue (frozen) from at least 6 female hamsters per treatment group were used. Frozen tissues were quickly weighed and suspended in 1 ml of cold buffer (5 mM Tris/ 5 mM EDTA, pH 7) containing 10μg/ml of the following proteinase inhibitors: benzamidine, soybean trypsin inhibitor, and leupeptin (Sigma; St. Louis, MO). Tissue was homogenized at 8,000 to 10,000 RPM with a tissue homogenizer (Tissue Tearor; model 985-370; Biospec Products) for 10 seconds, re-suspended in 5/5 buffer, and centrifuged at 19,000 rpms for 10 minutes at 4°C. Supernatant was removed by simple decanting. Pellet was resuspended in cold 75/5/2 buffer (75 mM Tris/ 5 mM MgCl2/ 2 mM EDTA, pH 7) with proteinase inhibitors as in 5/5 buffer. Pellet was resuspended in a μl volume equal to 10 times the original weight of tissue in mg. This mixture was vortexed by vigorous pipetting and kept on ice. The membrane/ buffer suspension was then filtered through a nylon mesh filter (Spectrum, Microgon Spectra/ Mesh macroporous filter; Laguna Hills, CA) into a cold conical tube. A 100 μl sample was taken and saved for protein evaluation. The remaining membrane mixture was aliquoted as approximately 500 μl samples were into cold 1 ml cryo tubes, snap frozen in liquid nitrogen, and stored at -80°C until further use.

Similarly for cell lines, cultured cells were grown to confluency in T-75 ml culture flasks. Then cells were washed with PBS. 10 mls of cold 5/5 lysis buffer was added to the flasks. The cells were collected into the cold buffer by scrapping the bottom of the culture

flask with a disposable cell scraper (FisherBrand).

Cell suspensions were centrifuged at 19,000 RPM (same as the fetal tissues) for 10 minutes at 4°C. The supernatant was removed by decanting and the pellet was resuspended in 10 mls of cold 5/5 lysis buffer and kept cold. This mixture was homogenized for 8-10 seconds using a tissue homogenizer as with the fetal tissues. The mixture was re-centrifuged at 19,000 RPM (same as the fetal tissues) for 10 minutes at 4°C. The supernatant was removed by decanting. The pellet was resuspended in 1.0 ml cold 75/5/2 buffer. A 100 μl sample was taken and saved for protein evaluation. The remaining membrane mixture was aliquoted as approximately 500 μl samples were into cold 1 ml cryo tubes, snap frozen in liquid nitrogen, and stored at -80°C until further use.

IV. Protein Evaluation

Protein standards were prepared in 75/5/2 buffer (see above section) using an albumn standard (bovine serum albumin, fractionV; purchased as vials with 2.0 mg/ml in a 0.9% aqueous NaCl solution containing sodium azide; Pierce ;Rockford, Illinois). Concentration of standards prepared were 0 μg, 10 μg, 20 μg, 40 μg, 60 μg, 80 μg, 100 μg, and 120 μg. BCA Protein Assay Reagent was prepared by mixing 1 part Reagent B (Pierce; Rockford, Illinois) to 50 parts Reagent A (Pierce; Rockford, Illinois). To 12 x 75 mm borosilicate glass culture tubes was added 100 μl of each standard and 100 μl of the unknown protein samples to be analyzed. To these 100 μl samples was added 2 ml of the BCA Protein Assay Reagent. Sample was vortexed after addition of the Reagent. Samples were incubated at 37°C for 30 minutes. Absorbances of each standard was read in cuvettes at 562 nm using a quantitative

program on UV-visible light recording spectrophotometer (Shimadzu Corporation; Kyoto, Japan) The spectrophotometer generates a standard curve. The absorbance of each unknown is read in a similar manner. The spectrophotometer generates protein concentrations in μg/100 μl for each unknown based on the standard curve. Buffer (75/5/2) was used as a blank.

V. Saturation Binding Assays

In brief

These assays were performed in a total volume of 250 μl containing 5 μg of protein at room temperature for a 45 minute incubation period. The selective ligand for β-adrenergic receptors [^{125}I]-(-)Iodocyanopindolol (CYP) (NEN) was used at concentrations ranging from 2 pM to 220 pM to establish a saturation curve. Specific binding was calculated by subtracting binding in the presence of an excess concentration (100 mM) of the β-specific antagonist Alprenolol (T.Cookson). The assay was performed with triplicate samples with each data point in the saturation isotherm representing the mean value of the triplicate samples. The assay was terminated by the addition of 2 ml of cold 10 mM Tris buffer and harvesting of the membranes on to Whatman GF/C filters by vacuum filtration with a

Brandel Cell Harvester. The filters were washed 3 times with 2-3 ml of cold 10 mM Tris buffer and radioactivity was counted on a Packard gamma counter.

Basic procedure

The assays were performed in 12 x 75 mm borosilicate glass tubes (Fisher Scientific). There were 6 tubes per assay set. Each assay set represented a given assay concentration of radioligand. The tubes were labeled according to radioligand concentration. The first triplicate in a given set were for total binding and contained radioligand and ultrafiltrated sterile water. The second triplicate in a given set were for evaluation of nonspecific binding and contained radioligand and alprenolol at 100 mM.

Five or 10 μl of the stock radioligand (CYP) solution was counted to determine cpm/ μl (counts for the day) and take into consideration any decay. This valve was used to prepare dilutions of CYP: Prior to making dilutions, theoretical valves of cpm/ 25 μl and cpm/ μl were determined for each concentration of CYP to be used. The amount of CYP to be used for a given dilution was determined using the following formula:

$$\frac{\text{(Total volume of isotope solution in } \mu\text{l) X (theoretical cpm for given concentration/}\mu\text{l)}}{\text{Counts for the day in cpm/}\mu\text{l}}$$

The dilutions were prepared in 75/5/2 buffer with proteinase inhibitors (see membrane preparation section). After preparing dilutions, 25 μl of each dilution was counted in a gamma counter. Concentrations (generally in pM) were determined for each cpm valve. These dilutions were protected from light until ready for use.

A stock concentration of Alprenolol was prepared so that 25 μl of that concentration

would give a final concentration of 100 mM in a final assay volume of 250 μl. The solution was made using ultrafiltrated sterile water.

The final item prepared on for an assay was the membrane solution. The membrane solution was prepared in 75/5/2 buffer with proteinase inhibitors (see above membrane preparation section) for a volume of 200 μl for each assay tube. Total number of tubes in the assay plus 20% was multiplied by 200 to determine total volume of membrane solution needed. Based on the μg/ μl protein concentration determination, the volume of membrane preparation (final membrane preparation frozen at -80°, see above under membrane preparation) was calculated so each assay tube would contain 5 μg protein. This volume was added to a cold 50 ml conical tube on ice. The total volume of membrane solution was subtracted from the volume of membrane protein added to determine volume of 75/5/2 buffer (with proteinase inhibitors, see above section) needed.

A number of enzyme/metabolic inhibitors were added to the membrane solution. Serotonin hydrochloride (Sigma) at an assay concentration of 10 uM was used to block potential binding of [^{125}I] CYP to serotonin receptors. Pargyline hydrochloride (Sigma) was used at an assay concentration of 10 uM to block serotonin metabolism. Ascorbic acid (Sigma) was used at an assay concentration of 1 mM. Ascorbic acid inhibits enzymatic break down of epinephrine and norepinephrine was used in the competition assays and was used here purely to keep assay conditions constant.

For assay, 200 μl of membrane solution was added to all tubes. Then 25 μl of ultrafiltrate water was added to the first triplicate (total binding). Then 25 μl of Alprenolol was added to the second triplicate. Finally, 25 μl of CYP was added to all tubes. The rack

containing the tubes was placed on a standard vortexer to lightly vortex the contents of all assay tubes. The rack was placed in a shaking water bath at room temperature for 45 minutes.

Assays were terminated by harvesting onto a GFC glass filter (Brandel; Gaithersburg, MD) using a Brandel Harvester (model MP-48R; Gaithersburg, MD). To separate bound from unbound, filters were washed three times with 1-2 ml of cold 10 mM Tris hydrochloride, pH 7.5. Corresponding filters were placed into labeled tubes and capped. Radioactivity of ^{125}I was counted using a Packard gamma counter.

VI. Competition Binding Assays

In brief

The assays were performed in triplicate with a total volume of 250 μl containing 5 μg of protein at room temperature in a shaking waterbath with a 30 minute preincubation period for membranes and cold ligand and a 45 minute incubation period for assay. Ascending concentrations of agonists (norepinephrine and epinephrine; T.Cookson), subtype selective antagonists (atenolol, β_1 and ICI 118, 551, β_2; T. Cookson) , and NNK (Chemsyn Science Laboratories) competed with a single concentration of [^{125}I] CYP for β-adrenergic binding sites. The assay was performed with triplicate samples with each data point in the competition isotherm representing the mean value of the triplicate samples. Nonspecific binding was measured in the presence of excess concentrations (100 μM) of alprenolol (T. Cookson). The assay was terminated by the addition of 2 ml of cold 10 mM Tris buffer and

harvesting of the membranes on to Whatman GF/C filters by vacuum filtration with a Brandel Cell Harvester. The filters were washed 3 times with 2-3 ml of cold 10 mM Tris buffer and radioactivity was counted on a Packard gamma counter.

Basic procedure

The assays were performed in 12 x 75 mm borosilicate glass tubes (Fisher Scientific). There were 3 tubes per assay set. Each assay set represented a triplicate of a given assay concentration of unlabeled competing ligand. The tubes were labeled according to competing concentration. A final triplicate was added to measure nonspecific binding and consisted of alprenolol as the unlabeled competing ligand.

Five or 10 μl of the stock radioligand (CYP) solution was counted to determine cpm/ μl (counts for the day) and take into consideration any decay. This valve was used to prepare a dilution of a 300 pM CYP. Prior to making this dilution, a theoretical valve of cpm/ 25 μl and cpm/ μl was determined for this concentration of CYP to be used. The amount of CYP to be used for a this dilution was determined using the following formula:

$$\frac{\text{(Total volume of isotope solution in } \mu\text{l) X (theoretical cpm for given concentration/}\mu\text{l)}}{\text{Counts for the day in cpm/}\mu\text{l}}$$

The dilution was prepared in 75/5/2 buffer with proteinase inhibitors (see membrane preparation section). After preparing the dilution, 25 μl was counted in a gamma counter. The concentration was determined to make sure it was close (within 10%) of the target concentration. This dilution was protected from light until ready for use. The concentration of radioligand used in the competition studies with atenolol and ICI, 118,551 was 300 pM.

The radioligand concentration used in the competition studies with epinephrine, norepinephrine, and NNK in the untreated fetal pancreas was 112 pM. The concentration of radioligand used in the competition study with NNK in the ethanol treated fetal pancreas was 44pM.

Following the preparation of the CYP dilution. Dilutions were made for the competing ligand from an initial stock solution. Dilution concentrations were made to give specific resulting concentrations using a volume of 25 μl in a total volume of 250 μl.

Serial dilutions were prepared for the competing ligands to be used. Dilutions were prepared in sterile water from an initial stock solution which was made fresh on the day of the assay. In the preparations of NNK, the dilutions were kept warm (37°C) to insure compound remained in solution. Dilution concentrations used were those that would result in the desired assay concentrations added as a volume of 25 μl into a total volume of 250 μl. Table 1 (appendix B) lists the dilutions used and assay concentrations achieved.

The final item prepared for an assay was the membrane solution. The membrane solution preparation was the same as for the saturation binding assays (see above). The same enzyme/metabolic inhibitors were added to the membrane solution as in the saturation assays. In addition, to the ascorbic acid, serotonin hydrochloride, and pargyline, aminobenzotriazole (assay concentration of was used in assays with norepinephrine and epinephrine and metyrapone (a cytochrome p450) (Sigma) was used to assays with NNK at an assay concentration of 1uM to inhibit metabolism of NNK. The membranes with these inhibitors were kept on ice for 5 minutes prior to using in the assay.

For each assay, 200μl of membrane solution was added to all tubes. Then 25 μl of

appropriate concentration of competing ligand was added. The rack containing the tubes was placed on a standard vortexer to lightly vortex the contents of all assay tubes. The rack was placed in a shaking water bath at room temperature for 30 minutes. After the 30 minutes, the CYP was added to all tubes. The rack of tubes was again lightly vortexed and the rack was placed in a shaking water bath at room temperature for 30 minutes.

Competition assays were terminated the same as saturation assays by harvesting onto a GFC glass filter (Brandel; Gaithersburg, MD) using a Brandel Harvester (model MP-48R; Gaithersburg, MD). To separate bound from unbound, filters were washed three times with 1-2 ml of cold 10 mM Tris hydrochloride, pH 7.5. Corresponding filters were placed into labeled counting tubes and capped. Radioactivity of ^{125}I was counted using a Packard gamma counter (model Cobra 5005; Meriden, CT).

VII. Computer Analysis of Binding Data

Saturation and competition binding data was analyzed using a software package called Prizm (GraphPad Software; San Diego, CA). This software contains built-in programs for analyzing radioligand binding data.

Saturation binding data analysis

Triplicate values in cpm were entered for each given concentration of radioligand. The values in cpm for total binding (radioligand binding in presence of water) were placed in one column set (as Y values) for each corresponding radioligand concentration (X values) (given in molar units). In a similar manner, the values in cpm for nonspecific binding

(radioligand binding in presence of excess unlabeled ligand) were placed in the adjacent column set. Counts per minute represented cpm/ 5 mg. The cpm/ 5 mg was converted to cpm/mg by transforming the data using the equation Y*K where K equals the factor 200. The data was further transformed from cpm/mg to fmole/mg by transformation using the equation Y/K where K equals the factor 3418.8. Radioligand concentrations were transformed from molar units to log molar units. The computer generated means of each triplicate and plotted the data in a graph.

For specific binding, the computer subtracted the means for the total and nonspecific binding counts at each given concentration of radioligand. This data is plotted on the same graph as for the total and nonspecific data.

Curves were generated by the computer for total, specific, and nonspecific by fitting the data using nonlinear regression. This analysis also calculates K_d (affinity of the radioligand) and B_{max} (number of receptors).

Competition binding data analysis

Triplicate valves in cpm (Y values) were entered for each given concentration of competing ligand (X values). Radioligand concentrations were transformed from molar units to log molar units. The computer generated a mean cpm value for each triplicate and plotted the data in a graph.

The graph was examined to determine the concentration at which the competing ligand began to compete with the radioligand (i.e. at which point the competition curve started to drop). This information was used to further transform the data to reflect the

percent binding of the radioligand at any given concentration.

Competition curves were fitted to the data using nonlinear regression. The data was examined to determine whether a one-site or a two-site competition model was more appropriate. The computer program generates an EC_{50} (concentration of the receptor that competes for half the specific binding) and K_i (affinity of the competing ligand for the receptor).

VIII. Molecular Studies

RNA isolation

RNA was isolated from fetal pancreatic tissues and human pancreatic adenocarcinoma cell lines using guanidine isothiocyanate (GIT)/ cesium chloride and overnight centrifugation (MacDonald, 1987).

A GIT buffer solution was prepared by mixing 47.2 g GIT and 1 ml 3M sodium acetate (pH 7) to a final volume of 100 ml of sterile water. The final GIT concentration was 4M. The GIT buffer solution was sterile filtered through a disposible tissue culture filter unit with a 1 mm filter (Nalgene). A CsCl buffer solution was prepared by mixing 95.97 g CsCl (final concentration 5.7M), 1 ml 3M sodium acetate (pH 7), and 2 ml 0.5M EDTA (pH 8) with sterile water to a final volume of 100 ml. The CsCl buffer solution was sterile filtered through a 0.8 μm filter.

Pancreatic fetuses from 3 pregnant hamsters from two groups were utilized. (Tissue had been harvested previously and stored; see transplacental hamster model in materials and methods section of this chapter.) The frozen tissue was weighed. (Resulting weights were

0.235 g for nontreated pancreases and 0.314 g for ethanol treated pancreases.) Tissue of a given treatment was placed in a mortar and pestle with liquid nitrogen and ground to a powder. Ground tissue with remaining liquid nitrogen was added to a sterile conical tube. Following the evaporation of the liquid nitrogen, 8 ml of GIT was added to the conical tube with the tissue. The ground tissue in GIT was homogenized at 25,000-30,000 RPM using a tissue homogenizer (Tissue Tearor, BioSpec Products).

For cultured cell lines, cells were scraped from bottom of flask and well mixed into the media within the flask. Media and cells were transferred to a sterile 50 ml conical tube and centrifuged at 1800 RPM for 3 minutes. The media above the cell pellet was discarded. The pelleted cells were resuspended in 25 ml of PBS and re-centrifuged at 1800 RPM for 3 minutes. The PBS was discarded. The cell pellet was dissolved in 8 ml of GIT.

The remaining procedures were the same for both tissue and cultured cell lines. The resultant fluid (after addition of GIT) was then drawn and aspirated several times through a 20 g needle attached to a 30 ml syringe. A Seton centrifuge tube (Seton Scientific; Los Gatos, CA) was prepared for each sample by adding 4 ml of CsCl buffer to each tube needed. The sheared cell solution was added to the centrifuge tube by expelling the solution from the 30 ml syringe along the inner wall of the centrifuge tube just above the level of the CsCl. GIT was added until the fluid level reached the very top of the tube. The Seton tubes were placed in a swinging bucket rotor in a balanced arrangement and centrifuged at 30,000 RPM (111,000 G) at 20°C for at least 23 hours.

Following centrifugation, each tube was carefully removed under a designated RNA hood. The supernatant was removed by aspiration using an autoclaved RNA pipette. Fluid

was removed until reaching 0.5 cm of the tube base. This 0.5 cm base was removed from the remainder of the tube with a sterile razor blade. The remaining supernatant was removed using a P-1000 pipette with an RNA certified tip, being careful not to disturb the small translucent RNA pellet at the bottom of the tube base. The pellet was resuspended in 300 μl of 0.3M sodium acetate by pipetting up and down and scraping sides of tube with pipette tip. The resuspended RNA was transferred to a sterile 1 ml Eppendorf tube on ice. The Seton tube base was re-washed using 100 μl of 0.3M sodium acetate. This remaining fluid was transferred to the 1 ml Eppendorf tube on ice. The 1 ml Eppendorf tube was gently flicked to mix contents. The RNA within the 1 ml Eppendorf was precipitated by adding 100 μl of 100% ethanol and placing the sample in a freezer at -80°C for at least 15 minutes. The frozen precipitated RNA was then centrifuged in microcentrifuge at 14,000 RPM at 4°C for 10 minutes. The supernatant was aspirated. The pellet was washed by the addition of 100 μl of 80% ethanol and re-centrifuging at 14,000 RPM at 4°C for 5 minutes. The supernatant was aspirated. The RNA pellet was resuspended in 50μl of certified Dnase/RNase free water (Promega; Madison, WI).

To measure the RNA concentration, 3 μl of the RNA sample was added to an quartz cuvette containing 297 μl of nuclease free water. The RNA was gently mixed by placing parafilm over the top of the cuvette and gently inverting several times. The concentration was determined by measuring the optical density (OD) at 260 nm and 280 nm using a Photometric program on a UV-visible light recording spectrophotometer (model UV-160; Shimadzu Corporation; Kyoto, Japan). Formula used to determine RNA concentration was as follows:

OD at 260 nm X dilution factor X OD constant for RNA

The dilution factor used was 100. The OD constant for RNA is 40.

RNA purification

RNA from all samples were purified of any potential contaminating genomic DNA using the DNase I technique described by Ambion (Austin, TX; Bauer, 1997; Dixon, 1998). The reaction was performed under a PCR (polymerase chain reaction) hood. The reaction utilized 2 μg of RNA (previously frozen). To the RNA was added 1μl of DNase I 10X buffer, 1μl of DNase I solution, and a volume of nuclease free water (certified Rnase/ DNase free water; Promega) to give a final reaction volume of 10 μl. The mixture was incubated for 15 minutes at room temperature. The reaction was terminated by the addition of 1μl of 25 mM EDTA to the tube followed by incubation for 10 minutes at 65 °C.

Reverse transcription (RT)

Purified RNA was reverse transcribed into complementary DNA (cDNA) using the technique described by Ambion (Austin, TX; Farrell, 1997; Farrell, 1993; Innis, 1990) using the RETROscript™ kit. Each RT reaction utilized the reaction tube and contents from the RNA purification step (above section) with 2 μg of RNA, 1μl of DNase I 10X buffer, 1μl of DNase I solution, and a volume of nuclease free water (certified Rnase/ DNase free water; Gibco) in a final volume of 10 μl. To this purified RNA solution was added 2μl of oligo(dT) 12-18 primers (Gibco; Gaithersburg, MD; supplied at a concentration of 0.5 mg/ml). The solution was incubated for 3 minutes at 82 °C and then immediately placed on ice. To this

mixture, 200 U (1μl) Moloney-mouse leukemia virus reverse transcriptase (Gibco), 40 U (μl) Rnasin ribonuclease inhibitor (Promega), 2 μl 10X buffer (100 mM Tris-HCl pH 8.3, 500 mM Kcl, 15 mM MgCl2; Ambion), 10 mM (2μl) DTT, and 0.5 mM (1 μl) each of dGTP, dATP, dCTP, and dTTP for a final volume of 20 ul. The reaction mix was incubated at 37°C for 1 hour and then heat inactivated for 10 minutes at 92°C. A negative control reaction was prepared in the same way with 1 μl nuclease free water (Promega) instead of M-MLV reverse transcriptase. The final RT product was either frozen at -20°C until use or immediately used for the polymerase chain reaction (PCR).

Polymerase chain reaction

The amplification of cDNA was accomplished using the technique described by Ambion (Austin, TX; Farrell, 1997; Farrell, 1993; Innis, 1990) using the QuantumRNA™ kit. Each PCR reaction utilized 5 μl of the 20 μl cDNA generated from RT reaction (above section). To this was added 2 μl (0.2 mM) dNTPs, 5 μl 10X PCR buffer (100 mM Tris-HCl ph 8.3, 500 mM Kcl, 15 mM MgCl2), 0.25 μl (1.25 U) SuperTaq polymerase (Ambion), 2.5 μl (5%) DMSO (molecular grade; Sigma). Each reaction received either 7 or 5 μl of a gene specific primer mixture (5 mM forward/ 5 mM reverse) and/or 0.75 μl of a cyclophilin primer mixture (provided by Ambion, used as an internal control for amplification of a moderately expressed "housekeeping" message). A volume of nuclease free water was added to bring the final reaction volume to 50 μl. Primers used for human β_1 and β_2 adrenergic receptors were obtained from published sources (β_1, Ihl-Vahl, 1995; β_2, Fugi, 1997). The forward and reverse primers for β_1 were CAA GTG CTG CGA CTT CGT CAC

C and GCC GAG GAA ACG GCG CTC, respectively, and resulted in a 159 base pair product. The forward and reverse primers for β_2 were ACG CAG CAA AGG GAC GAG and CAC ACC ATC AGA ATG ATC AC, respectively, and resulted in a 401 base pair product. Cyclophilin internal control produced a 216 base pair product. For control purposes, RT-PCR was also completed with RNA derived from Chinese Hamster ovary lines transfected with the human β_1 (Rex50 cell line) and β_2 (NBR29 cell line) genes.

RT/PCR products, including controls, were visualized using agarose gel electrophoresis. A 2% agarose gel was prepared by adding 2 g of regular agarose to 100 ml of TBE (1X). The agarose was dissolved in the TBE by microwaving for 3 minutes until the solution was just beginning to boil. Mixture was gently swirled and slightly cooled. The agarose gel was supplemented with ethidium bromide at a concentration of 1μl ethidium bromide per 10 ml of agarose gel solution prepared. Gel lanes were loaded with a mixture of 20μl of PCR product and 2μl loading buffer (5Prime-->3Prime, Inc.; Boulder, CO). A marker lane was loaded with a mixture of 3.0 μl of a 100 base pair DNA ladder (Gibco), 1 μl loading buffer, and 7 μl water. Gel electrophoresis was performed at 75 volts for 2 hours. The bands were visualized with ultraviolet light using a UVP GDS 7500 (Upland, CA). Bands to be sequenced were sliced from the gel with a clean razor blade while visualizing under UV light. The DNA bands were purified of primers using the GENECLEAN kit from BIO 101(LaJolla, CA).

DNA sequencing

Sequencing was performed by Dr. Neil Quigley at the University of Tennessee Molecular Biology Resource Facility. The DNA sequencing was performed with an ABI Prism Dye Terminator Cycle Sequencing reaction kit (Perkins-Elmer, Inc.; Foster City, CA) on an ABI 373 DNA sequencer Initial (Perkins-Elmer, Inc.).

Sequence data text files were compared with the same data displayed in four-color electropherograms and then edited. Sequences were compared to previously published GenBank sequences for human β_1 and β_2 -adrenergic genes and percent homology was determined using the DNASIS program.

Chapter 3: Results

All figures and tables are contained in appendices A and B, respectively, following the reference section of this part of the dissertation.

I. Saturation binding studies

Total and nonspecific binding was measured in the presence of increasing concentrations of [^{125}I]CYP and a single excess concentration of alprenolol. The generated curves for untreated fetal pancreas membrane vesicles (figure 3) and for ethanol treated fetal pancreas membrane vesicles (figure 4) represent nonlinear regression isotherms. The calculated Bmax values for the untreated and the ethanol treated fetal pancreas were 233 fmole/mg and 328 fmole/mg, respectively. The calculated KD values were 123.6 pM (untreated) and 173.2 pM (ethanol treated). The findings are suggestive of either an increase in receptor number (upregulation) or an increase in receptor sensitivity to its agonists (sensitization). An unpaired t-test with Welch's correction for unequal variances showed the receptor numbers were not statistically significant.

II. Competition binding studies

The percent displacement of [^{125}I]CYP by the competing ligand was measured in the presence of a single concentration of the radioligand with increasing concentrations of the competing drugs. The generated curves represent nonlinear regression isotherms for one or

two classes of binding sites. Equation models were assessed for the best fit based on R_2 values (a measure of goodness of fit). In addition to R_2 values, the computer program used generated EC_{50} values (EC_{50}-1, high affinity site; EC_{50}-2, low affinity site) and K_i values for each isotherm. EC_{50} represents the effective concentration of competitor ligand that blocked 50% of the binding of the radioligand. K_i represents a measure of the affinity of the receptor for the ligand. The figures for all generated receptor binding curves are contained within the appendix. The legend for each figure states the EC_{50}, K_i, and R_2 values for each curve.

Competition assays using norepinephrine and epinephrine was completed in the untreated fetal tissue only. Epinephrine and norepinephrine are potent agonists for β-adrenergic receptors. Epinephrine binding to β_2-receptors is 10-50 more potent than norepinephrine. Norepinephrine and epinephrine exhibit about equal binding potency to β_1-receptors. The generated curves for epinephrine and norepinephrine are shown in figure 5. Both of these curves represent isotherms for 2 classes of binding sites. Although an agonist or antagonist may be site selective, a given competitor can bind to more than one subtype but bind to each subtype at differing proportions. Norepinephrine and epinephrine effectively competed against the radioligand for binding sites. At the high affinity site, both epinephrine and norepinephrine bind with equal affinity and displace at the same proportion. Since epinephrine is a more potent binder of β_2 than norepinephrine, binding at the high affinity site likely represents binding at β_2-adrenergic receptors. Approximately 25% of the receptors appear to be β_2 receptors. At the low affinity site, epinephrine was a more competent competitor; its curve is shifted just to the right of norepinephrine. The EC_{50} values (and respective K_i values) for high and low affinity binding sites in the epinephrine

curve were 561.9 pM (295.2pM) and 6.88 mM (3.6 mM), respectively. These values for norepinephrine were 461.1 pM (243.9 pM) and 25.6 mM (13.4 mM), respectively. Although epinephrine is the more competent competitor at the low affinity sites, these sites likely represent the proportion of β_1 receptors. This data essentially demonstrates the presence of both β_1 and β_2 subtypes in the untreated hamster fetal pancreas.

Competition curves with selective antagonist were completed in fetal pancreas derived from both treated and untreated dams. β_1 and β_2 subtypes were identified in both untreated and ethanol treated pancreas. In the untreated pancreas (figure 6) in the presence of ICI 118,551, a β2 selective antagonist, the EC_{50} values (and respective K_i values) for high and low affinity binding sites were 188 pM (53.8 pM) and 154 μM (44 μM), respectively. These values in the ethanol treated pancreas (figure 7) were 1.9 μM (672 nM) and 1.8 mM (644 μM). In the untreated pancreas in the presence of atenolol, a β_1 selective antagonist, the EC_{50} values (and respective K_i values) for high and low affinity binding sites were 63.4 pM (18.6 pM) and 154 μM (45 μM), respectively. In the ethanol treated pancreas, a curve was not generated for atenolol competition. Increasing concentrations of atenolol did not result in effective competition with the radioligand. The data in the untreated fetal pancreas confirms the data from the epinephrine and norepinephrine. β_1 receptors make up approximately 60% to 70%, and the proportion of β_2 is approximately 25 to 35%. These results demonstrate that untreated fetal pancreascontained both β_1 and β_2. However, ethanol treatment resulted in a change in the proportions of β_1 to β_2 subtypes. There was a decrease in the proportion of β_1 and an increase in the proportion of β_2 with β_2 representing slightly higher proportions. The curve for ICI 118,551 generated in the ethanol treated pancreas is

shifted slightly to the right of the curve generated in the untreated pancreas. This may in part be due to the higher number of receptors in the ethanol treated pancreas as demonstrated by the saturation curves and the need for higher concentrations of the competitor.

Figures 6 and 7 also show the isotherms generated when increasing concentrations of NNK were allowed to compete for binding sites. Although competition with NNK was performed at 40 pM, comparisons can be made in the EC_{50} values because the computer analysis takes into consideration the radioligand concentration when computing the EC_{50} and K_i. In the untreated pancreas, generated EC_{50} values (and respective K_i values) for high and low affinity binding sites were 154 nM (44 nM) and 123 mM μM (32.5 mM), respectively. These values in the ethanol treated pancreas (figure 7) were 32.3 nM (25.6 nM) and 27.1μM (26.1μM). Ethanol treatment resulted in a shift in the competition curve for NNK to the left resulting in a lower concentration of NNK needed to effective compete for the binding sites at both the high and low affinity.

Results of studies in the human pancreatic cell lines BxPC-3, AsPC, Capan-1, and Panc-1 are shown in figures 8, 9, 10, and 11, respectively. In all cell lines, the relative proportions of $\beta 1$ and β_2 were similar. In this factor, the cell lines are similar to the findings in the ethanol treated pancreas. Although, the competitions in both the BxPC and the AsPC cell line the presence of atenolol, generated nonlinear regression isotherms that best fit a single class of binding sites, inspection of the curves reveal that at higher concentrations of atenolol the curves would likely represent isotherms for 2 classes of binding sites. In all cell lines, ICI 118,551 was the more effective competitor with smaller EC_{50} values than with atenolol, particularly in the BxPC and AsPC cell lines. In the BxPC cell line in the presence

of ICI118,551, generated EC_{50} values for high and low affinity binding sites were 162 nM and 65.7 μM, respectively. In the presence of atenolol, nonlinear regression generated a curve best fit for a single class of binding sites, and the EC_{50} value was 826 μM. In the AsPC cell line in the presence of ICI 118,551, generated EC_{50} values for high and low affinity binding sites were 9.3 μM and 1.8 mM, respectively. In the presence of atenolol, nonlinear regression generated a curve best fit for a single class of binding sites, and the EC_{50} value was 2.1 mM.

In general the curves generated in the human pancreatic adenocarcinoma cell lines resemble those generated in the ethanol treated pancreas. Although, effective competition with atenolol was achieved in these human pancreatic adenocarcinoma cell lines, the effective concentration of atenolol needed was quite high.

III. Results of molecular determinations (RT-PCR)

Complementary DNA (cDNA) made from messenger RNA (mRNA) was adequately amplified. The results of the RT-PCR studies confirm findings in the competition studies in that beta-adrenergic mRNA was detected for both β_1- and β_2- adrenergic receptors in treated and untreated fetal hamster pancreas (figures 12 and 13), and in all 4 human pancreatic adenocarcinoma cell lines (figures 14-17). In all tissues and cell lines, bands for β_2 were notably higher in intensity as visualized with ethidium bromide and UV light.

Sequences were obtained for β_1 and β_2 from gel-purified PCR products from ethanol treated (figure 20,β_1 and figure 27, β_2), untreated fetal hamster pancreas (figure 19,β_1 and figure 26, β_2), and in 4 human pancreatic adenocarcinoma cell lines (figures 21-24, 28-31).

Sequences were compared with published sequences for human β_1- and β_2 - adrenergic receptor genes and percent homology was determined. In tissues and cell lines, both β_1 and β_2 sequences matched within 95-100%. Figures 18 and 25 represent homology comparison of sequences obtained from the transfected chinese hamster ovary cells. Rex 50 cell line has the gene for β_1. Homology comparison of the sequence obtained in this cell line (figure 18) matched 100% to the human β_1-adrenergic receptor gene. NBR 29 cell line has the gene for β_2. Homology comparison of the sequence obtained in this cell line (figure 25) matched 99% to the human β_2-adrenergic receptor gene.

Chapter 4: Discussion

Beta-adrenergic receptors are present in fetal hamster pancreas and in human pancreatic adenocarcinoma cell lines (BxPC, AsPC, Capan-1, Panc-1). Both β_1 and β_2 subtypes were detected in the fetal pancreas and in the cell lines. The methodology used in this chapter is well-established (Lemoine, 1989; Lemoine, 1985; Pauwels, 1988; Delavier-Klutchko, 1984; Ihl-Vahl, 1995; Kaumann, 1985) and has been used successfully in this laboratory (Park, 1995). Radioligand binding procedures revolutionized research practices in pharmacological studies. The earliest use of these techniques occured in the 1960's and early 1970's (Lefkowitz, 1970; Roth, 1973). Based on the early research in the field of radioligand binding, it became evident that high affinity antagonists would be the best radioligands. Iodinated radioligands have been used extensive ly in β-adrenergic receptor binding studies. [^{125}I]CYP is an excellent radioligand for these purposes. It possesses nonselective high affinity for β-adrenergic receptors in and has very low nonspecific interactions (Hoyer, 1982). Radioligand binding experiments require the ability to separate free radioligand from bound ligand (radioligand associated with the biological preparation). Vacuum filtration using glass fiber filters is one of the most efficient and reliable methods and was the method chosen for this dissertation work.

The concentrations of [^{125}I]CYP used by investigators for competition studies varies. It is often based on saturation data and in many cases reflects the concentration that reflects 50% saturation of the binding sites by the ligand (Stadel, 1991). In the studies discussed in

this dissertation, [^{125}I]CYP was used at a high concentration to insure complete saturation. Therefore, the ED_{50} generated are artificially high given that higher concentrations of a competing ligand would be needed to effectively compete for the binding sites.

Prior studies evaluating receptor and functionality with ethanol treatment, used nonselective agonists and antagonists and did not attempt to distinguish between β_1 and β_2 (Al-Nakkash,1996). In the current studies, selective antagonists for β_1 (atenolol) and β_2 (ICI 118,551) were used in an effort to determine which subtypes were present, in what proportion, and if there were modifications of those characteristics with ethanol exposure. It would appear that the hamster fetal pancreas from dams not treated with ethanol possesses both β_1 and β_2 with β_1 representing a higher proportion. In the ethanol treated pancreas, atenolol did not effectively compete for binding sites. The curve for ICI 118, 551 was shifted to the right. These data suggest that the β_1 subtype is downregulated. In addition, given the saturation data which revealed higher numbers of receptors, although not statistically not significantly different, in the ethanol treated membrane preparations than in the untreated, the data further suggest that the numbers of β_2 receptors increased. The curve for ICI 118, 551 was shifted to the right which would suggests that the affinity both receptor subtypes decrease. However, the overall higher numbers of receptors could partially explain this apparent shift in the curve of ICI 118,551 since of higher concentration of the competing ligand would be needed to effectively compete for binding sites.

In the RTPCR studies, based on visualization of intensity of the generated bands, a higher concentration of β_2 mRNA , as opposed to β_1, was detected in both the ethanol treated and untreated pancreas. In addition, both treated and untreated fetal pancreas appeared to

have mRNA for β_1 and differences in levels, based on band intensity, appeared to be the same. The reason for this is not known. Based on the binding data, the increase in the mRNA for β_2 over that of β_1 was expected. However, one would expected an increase in the mRNA for β_1 in the untreated pancreas. The amount of mRNA expressed in a tissue does not necessarily need to correlate with the amount of protein (in this case number of functional receptors), although generally it is presumed to be the case. In addition, these studies were general RTPCR studies only designed to detect the presence of mRNA. Future studies in this laboratory will address whether there is a difference in the relative amounts β_1 and β_2 for the different treatment groups using a procedure known as relative RTPCR. In relative RTPCR, intensity of the gene specific cDNA products are compared with eachother following standardization with 18S (an internal control housekeeping gene). This standardization controls for experimental variation (i.e., the amount of starting material, pipetting errors, etc.). Therefore, this procedure allows for direct comparison of RTPCR samples.

One of the most important findings in these radioligand binding studies, in relation to the thesis topic, is the evidence that the generated curve for NNK shifted to the left in the ethanol treated fetal pancreas compared to the untreated. This shift is evidence at the high and low affinity binding sites. It is not completely clear from the assays whether NNK is binding to β_1 sites or β_2 sites. The assays were not designed to determine this fact. However, given the fact that there has been an apparent downregulation of the β_1 sites, NNK could be binding preferentially to β_2 sites. However, using a different study design, binding of NNK to either receptor could prehaps be demonstrated. In other words, NNK itself may

not have a preference for a certain subtype and this biologic response could be a function of tissue type. In addition, the following notion is perhaps equally tantilizing. There are higher number of binding sites in ethanol treated pancreas as evidenced by the saturation data, and as stated earlier, that could result in higher concentrations needed for a competing ligand to effectively compete. However, in the case of NNK this factor did not appear to limit its ability to compete. Although, the concentrations of radioligand used in the ethanol and untreated fetal pancreas studies were different, the computer analysis uses the KD (affinity of the radioligand for the receptor) and the concentration of radioligand used to compute the EC50 for a competing ligand in a competition study. In addition, the NNK curve is shifted to the left of the curve generated with ICI 118,551. It is potentially possible that with ethanol treatment that a receptor could be altered in such a way that affinity of a given ligand could be altered as well. Changes in the sensitivity of β-adrenergic receptors (desensitization-decreased sensitivity and sensitization-increased sensitivity) has been documented (Perkins, 1991). Furthermore, if that were the case and resulted in a higher affinity of a carcinogen for that receptor, this change would be significant in terms of carcinogenic potential.

In regard to the studies conducted in human pancreatic adenocarcinoma cell lines, the data obtained in this dissertation support previous findings (Al-Nakkash, 1996). The multiple human pancreatic adenocarcinoma cell lines studied were found to have β-adrenergic receptors. The previous study did not distinguish between subtypes. Based on the studies presented in this document utilizing subtype specific antagonist, these cell lines were found to possess both β_1 and β_2, with β_2 representing the predominant subtype. BxPC-3 cell line had the best competition results; this finding was also evident in the study

conducted by Al-Nakkash, et al (1996).

The RTPCR studies in these cell lines confirmed the findings of the radioligand binding studies on one level, mRNA for both subtypes were expressed. Although, as stated earlier, the expression of mRNA does not necessarily confirm the expression of the corresponding protein, this may explain why the bands for β1 were less in intensity to those for β2 in the untreated fetal tissues. Messenger RNA for the human β_1 and β_2-adrenergic receptor genes was expressed in each cell line. The band for β_2 was greater in visual intensity compared to β_1. Relative RTPCR will be conducted to determine differences.

The findings of beta-adrenergic receptors in the fetal pancreas and in the human pancreatic adenocarcinoma cell lines are important with respect to this thesis. If beta-adrenergic receptors are to contribute a functional role in any step of the carcinogenesis process, they need to be present in the tissue of interest. These studies presented in this document were designed primarily to demonstrate the presence of these receptors. Assays that determine adenylate cyclase activity as a function of cAMP formation are most often used to demonstrate functionality of beta-adrenergic receptors. The studies presented here suggest a certain amount of functionality in the sense that mRNA is transcribed from the appropriate genes and is apparently then translated into protein forming a receptor capable of binding receptor specific agonists and antagonists. The formation of cAMP may not be the only determinant of functionality of beta-adrenergic receptors. There is increasing evidence, particularly in the cardiovascular literature, that activatin of beta-adrenergic

receptors can activate a number of phospholipases, including phospholipase A_2 and phospholipase D (Borda, 1998; Ruan, 1997). The potential importance of this pathway will be discussed briefly in chapter 4 (final summary).

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APPENDIX A

FIGURES

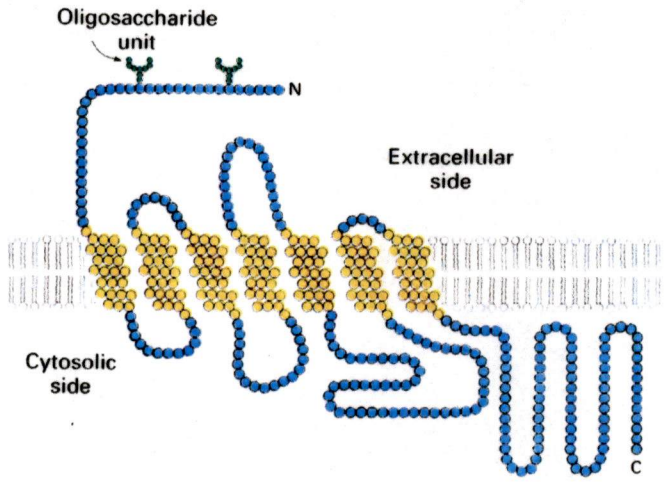

Figure 1: General structure of the β-adrenergic receptor. Figure taken from **Stryer, Lubert. *Biochemistry*, 4th ed** 1995:341 (**used without specific permission**). (Figure legend sites **Dohlman, HG, et al. Biochemistry 1987;26:2660.**) The structure has a basic seven-helix motif. The transmembrane helices are shown in yellow. *N*- oligosaccharide units, located on the extra-cytosolic side, are in green. A loop on the cytosolic side participates in the activation of the simulatory G protein, G_s.

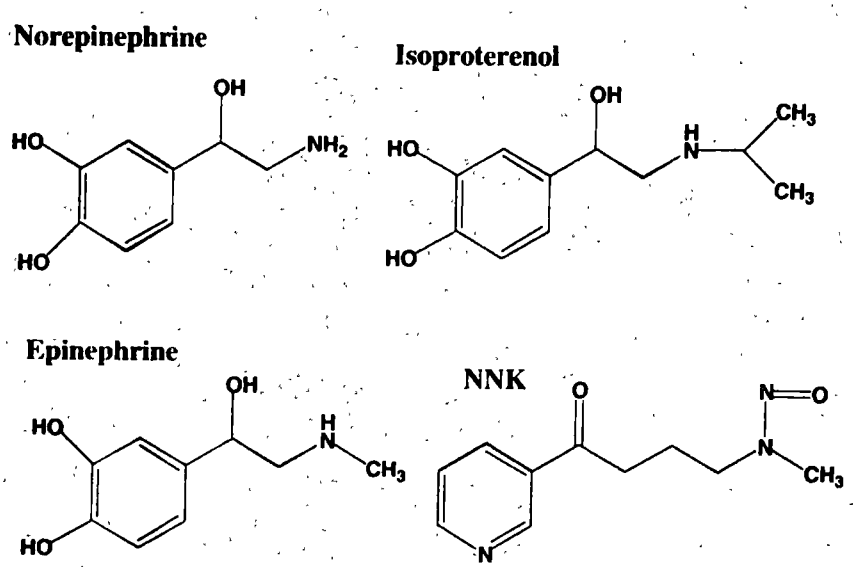

Figure 2: The catecholamines epinephrine and norepinephrine are the physiologic agonists for adrenergic receptors. Both of these neurotransmitters are comprised of a catechol ring with an aliphatic side chain containing a nitrogen atom. Epinephrine has a higher affinity to β-adrenergic receptors than norepinephrine. Increasing the bulk of the N-substitutions as in isoproterenol, and/ or lengthening the side chain, increases the selectivity for β-adrenergic receptors (Ruffolo, et al. J Med Chem 1994;38:3682). The intact catechol ring is a requirement for α-adrenergic but not β-adrenergic agonists. The heterocyclic ring of NNK with its alternating single and double bonds resembles the catechol ring. Like the catecholamines, NNK contains a nitrogen atom in an aliphatic side chain. The steric bulk of this nitrogen atom is increased by the *N*-nitroso group and a methyl group, making NNK a likely candidate for a β-adrenergic agonist.

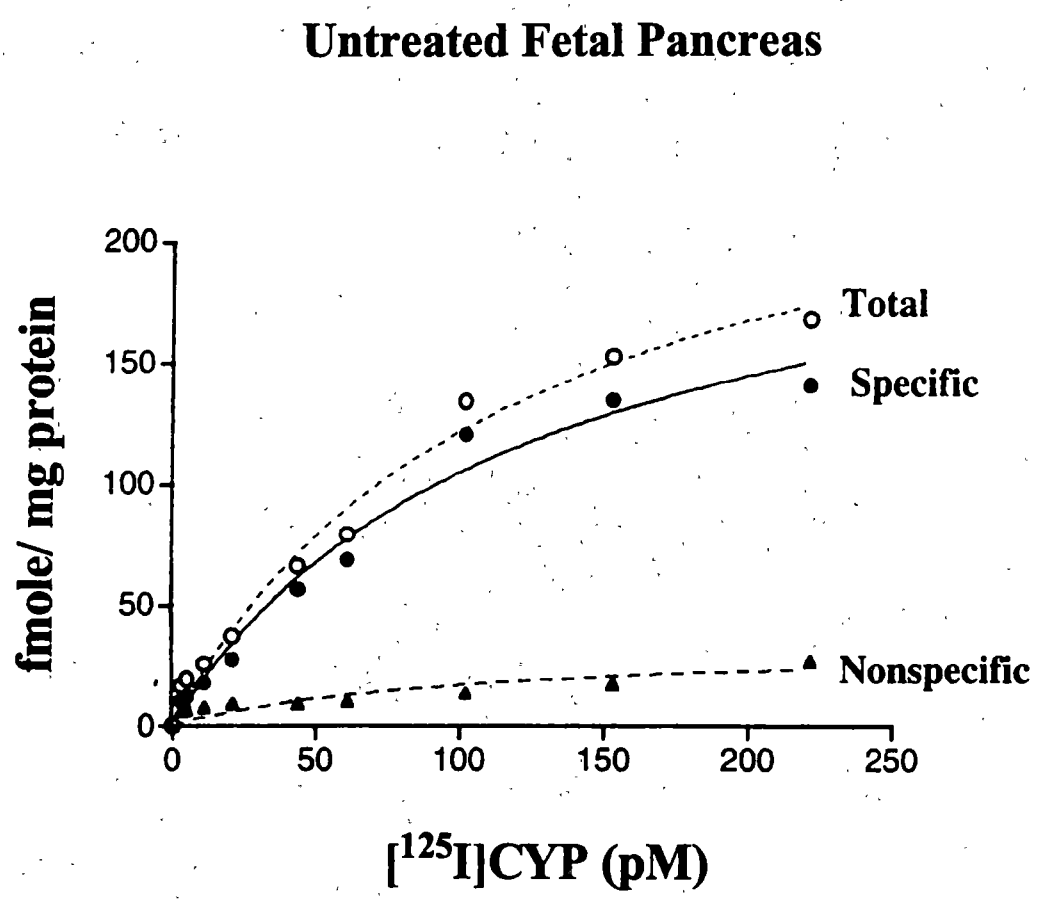

Figure 3: Saturation Curve of the selective β-adrenergic ligand [125I]-(-)-Iodocyanopindolol ([125I]CYP) in cell membrane fractions from **untreated fetal hamster pancreas.** Nonspecific binding was determined in the presence of the β-adrenergic ligand Alprenolol (100 mM). The curves represent nonlinear regression isotherms. B_{max} (measure of receptor numbers was 233 fmole/mg. K_D (affinity of the ligand for the receptor) was 123.6 pM.

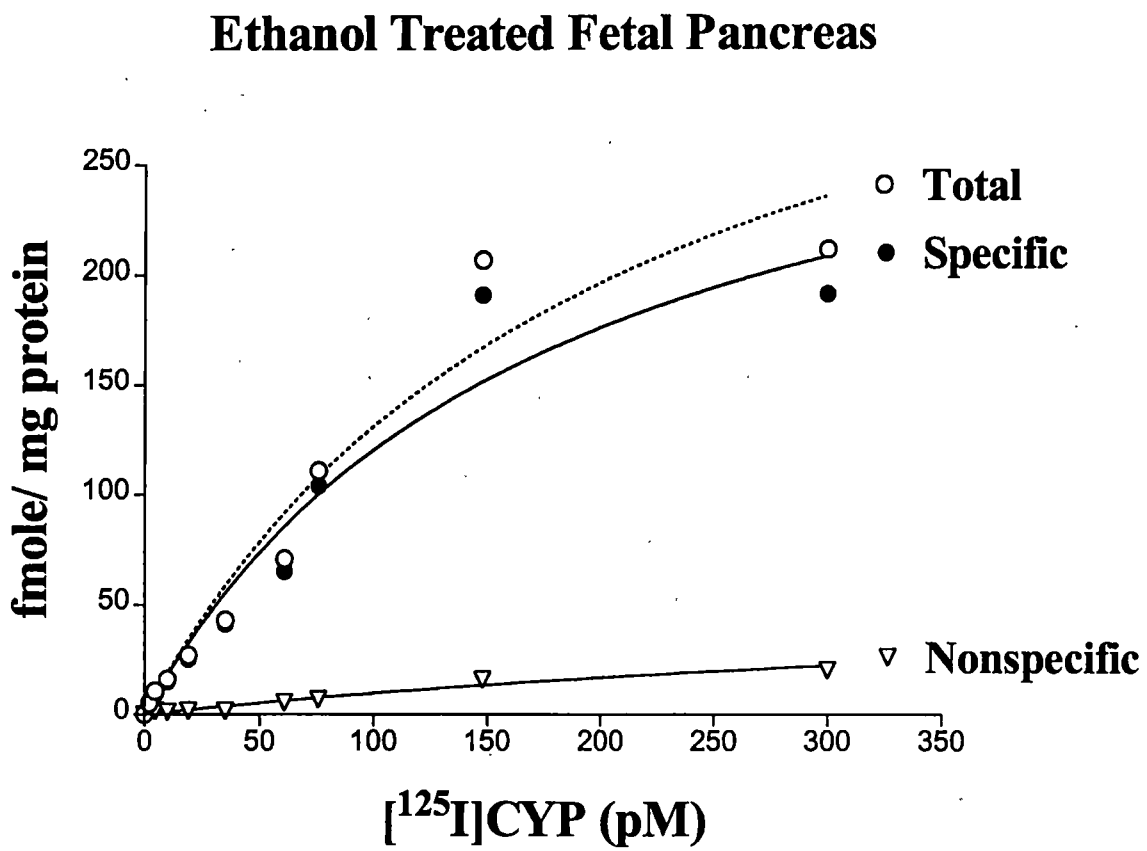

Figure 4: Saturation Curve of the selective β-adrenergic ligand [125I]-(-)-Iodocyanopindolol ([125I]CYP) in cell membrane fractions from **ethanol treated fetal hamster pancreas**. Nonspecific binding was determined in the presence of the β-adrenergic ligand Alprenolol (100 mM). The curves represent nonlinear regression isotherms. Ethanol treatment increased the overall receptor numbers from 233 fmole/mg (untreated pancreas) to 328 frmole/mg (ethanol treated pancreas). K_D changed from 123.6 pM (untreated pancreas) to 173.2 pM (ethanol treated pancreas).

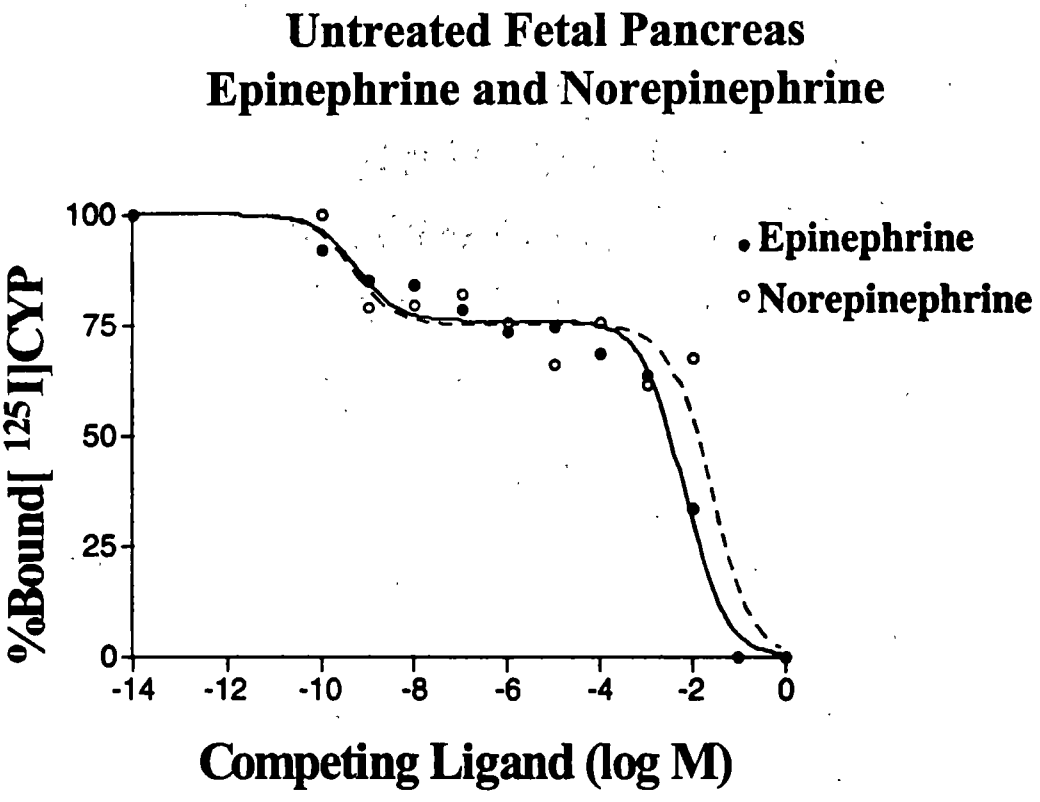

Figure 5: Results of receptor binding assays in which the neurotransmitters epinephrine and norepinephrine competed with [125I]CYP (112 pM) for β-adrenergic binding sites in cell membrane fractions from fetal hamster pancreas. Both competition curves represent nonlinear regression isotherms for two classes of binding sites. The EC_{50} values and affinities of the competing ligands were as follows: Epinephrine: EC_{50}-1: 561.9 pM, K_i-1: 295.2 pM, EC_{50}-2: 6.88 mM, K_i-2:3.6 mM; Norepinephrine: EC_{50}-1: 461.1 pM, K_i-1: 243.9 pM, EC_{50}-2: 25.6 mM, K_i-2: 13.4 mM.

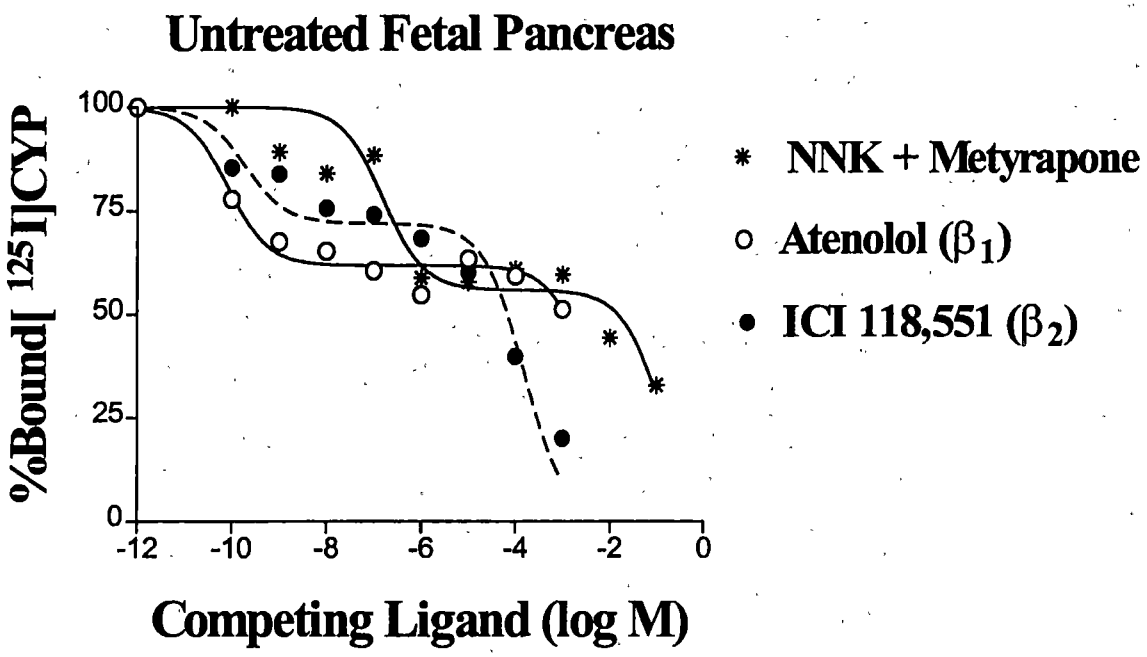

Figure 6: Results of receptor binding assays in which site selective ligands for β_1 (Atenolol) and β_2 (ICI 118,551) adrenergic receptors competed with [125I]CYP (300 pM) and NNK competed with [125I]CYP (112 pM) for β-adrenergic binding sites in cell membrane fractions from membrane fractions derived from **untreated fetal pancreas.** All three competition curves represent nonlinear regression isotherm for two classes of binding sites. The ligands bound high and low affinity sites. Ligand concentrations that blocked 50% of CYP binding (EC_{50}) and affinities (Ki) of the competing ligands for each class of binding sites were as follows: Atenolol EC_{50}-1: 80.8 pM, Ki-1: 32.1 pM, EC_{50}-2: 4.6 mM, Ki-2: 1.3 mM; ICI 118,551 EC_{50}-1: 188 pM, Ki-1: 53.8 pM, EC_{50} -2: 154 μM, Ki-2: 44 μM; NNK EC_{50}-1: 154 nM, Ki-1: 44 nM, EC_{50} -2: 123mM, Ki-2: 32.5 mM. The R2 values for the atenolol, ICI 118,551, and NNK curves were 0.95, 0.94, 0.88, respectively.

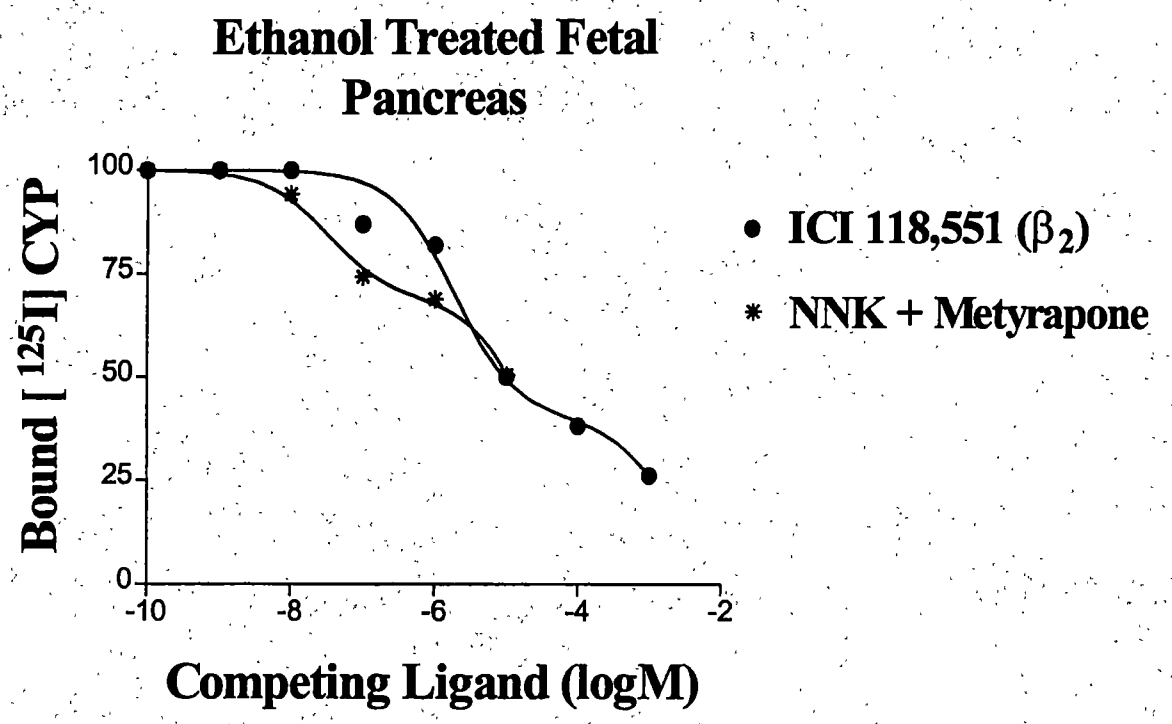

Figure 7: Results of receptor binding assays in which site selective ligands for β_1 (Atenolol) and β_2 (ICI 118,551) adrenergic receptors competed with [125I]CYP (300 pM) and NNK competed with [125I]CYP (44 pM) for β-adrenergic binding sites in cell membrane fractions from membrane fractions derived from **ethanol treated fetal pancreas**. The curve for Atenolol is not shown because it resulted in a horizontal line with no effective competition indicating the apparent amelioration of the β_1 subtype. Both ICI 118, 551 and NNKcompetition curves represent nonlinear regression isotherm for two classes of binding sites. Both ligands bound high and low affinity sites. Ligand concentrations that blocked 50% of CYP binding (EC_{50}) and affinities (Ki) of the competing ligands for each class of binding sites were as follows: ICI 118,551 EC_{50}-1: 1.9 μM, Ki-1: 672 nM, EC_{50}-2: 1.8 mM, Ki-2: 644 μM; NNK EC_{50}-1: 32.3 nM, Ki-1: 25.6 nM, EC_{50}-2: 27.1 μM, Ki-2: 21.6 μM. R_2 values for the ICI 118, 551 and NNK curves were 0.95 and 0.96, respectively.

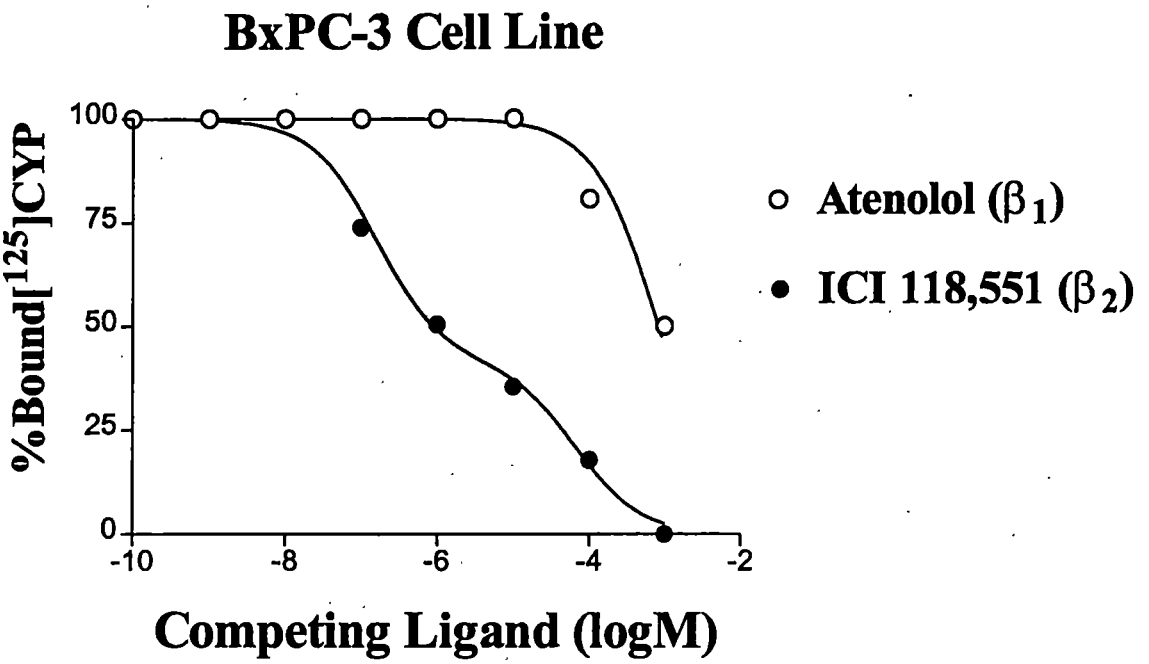

Figure 8: Results of receptor binding assays in which site selective ligands for β_1(Atenolol) and β_2 (ICI 118,551) adrenergic receptors competed with [125I]CYP (300 pM) for β-adrenergic binding sites in cell membrane fractions from the pancreatic carcinoma cell line **BxPC-3**. The competition curve for Atenolol represents a nonlinear regression isotherm for one class of binding sites. The competition curve for ICI 118,551 represents a nonlinear regression isotherm for two classes of binding sites. ICI 118,551 bound high and low affinity sites. The predominant β-adrenergic receptor type is interpreted to be β_2. Ligand concentrations that blocked 50% of CYP binding (EC_{50}) for each competing ligand for each class of binding sites were as follows: Atenolol EC_{50}: 826 μM; ICI 118,551 EC_{50}-1: 162 nM, EC_{50}-2: 65.7 μM. The R_2 value for the ICI 118,551 curve was 0.99. The R_2 value for the atenolol curve was 0.93.

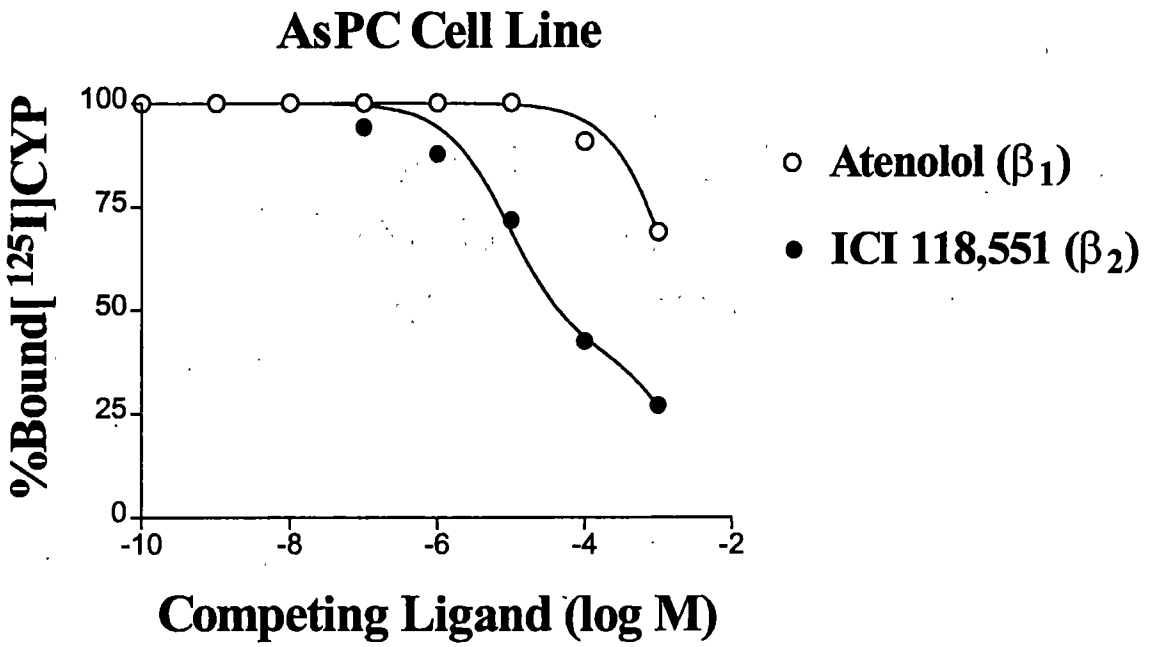

Figure 9: Results of receptor binding assays in which site selective ligands for β_1 (Atenolol) and β_2 (ICI 118,551) adrenergic receptors competed with [125I]CYP (300 pM) for β-adrenergic binding sites in cell membrane fractions from the pancreatic carcinoma cell line **AsPC**. The competition curve for Atenolol represents a nonlinear regression isotherm for one class of binding sites. The competition curve for ICI 118,551 represents a nonlinear regression isotherm for two classes of binding sites. ICI 118,551 bound high and low affinity sites. The predominant β-adrenergic receptor type is interpreted to be β_2. Ligand concentrations that blocked 50% of CYP binding (EC_{50}) for each competing ligand for each class of binding sites were as follows: Atenolol EC_{50}: 2.1 mM; ICI 118,551 EC_{50}-1: 9.3 μM, EC_{50}-2: 1.8 mM. The R2 value for the ICI 118,551 curve was 0.96. The R2 value for the atenolol curve was 0.78.

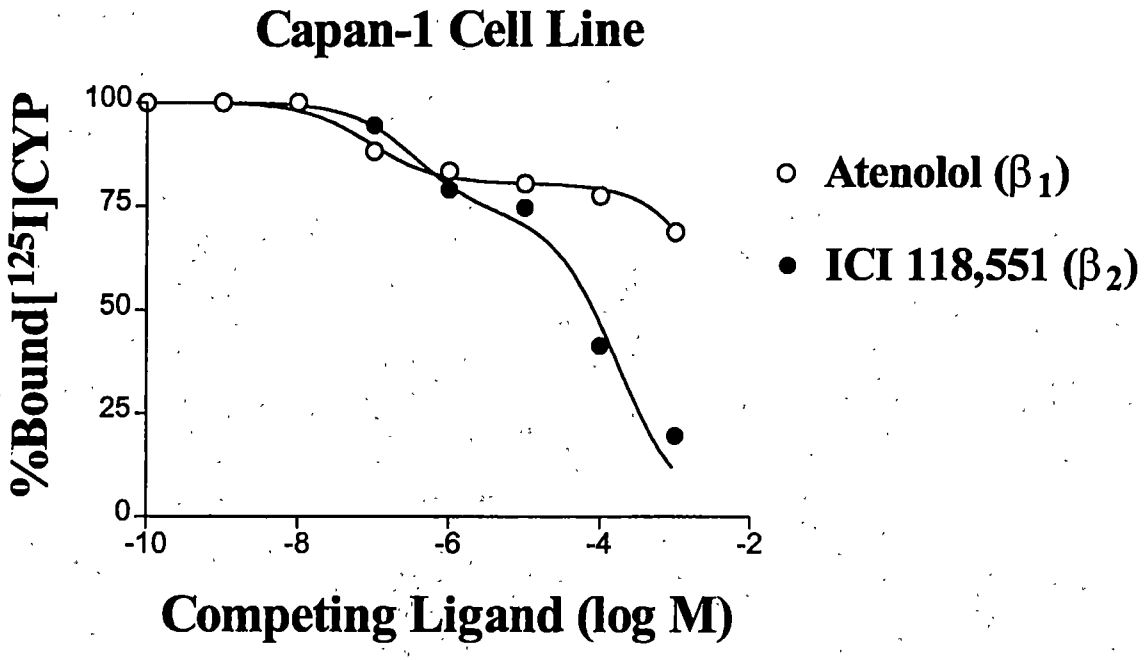

Figure 10: Results of receptor binding assays in which site selective ligands for β_1 (Atenolol) and β_2 (ICI 118,551) adrenergic receptors competed with [125I]CYP (300 pM) for β-adrenergic binding sites in cell membrane fractions from the pancreatic carcinoma cell line **Capan-1**. Both competition curves represent nonlinear regression isotherm for two classes of binding sites. Both ligands bound high and low affinity sites. Ligand concentrations that blocked 50% of CYP binding (EC_{50}) for each competing ligand for each class of binding sites were as follows: Atenolol EC_{50}-1: 86.8 nM, EC_{50} -2: 5.8 mM; ICI 118,551 EC_{50}-1: 354 nM, EC_{50}-2: 174 μM. The R_2 value for the ICI 118,551curve was 0.97. The R_2 value for the atenolol curve was 0.92.

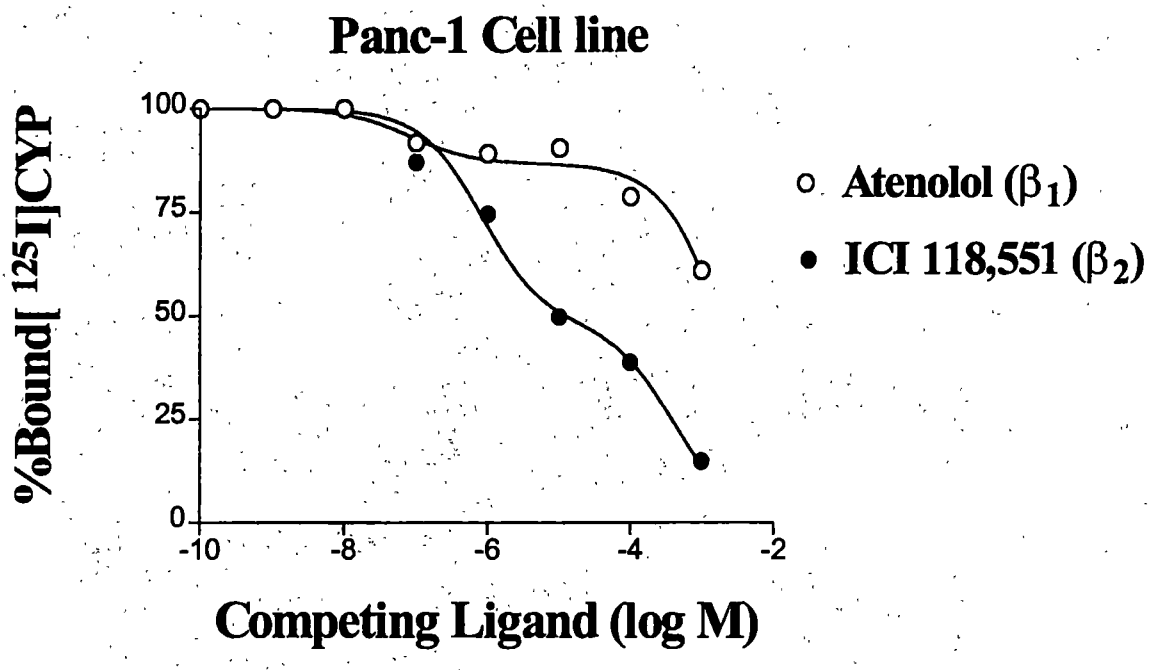

Figure 11: Results of receptor binding assays in which site selective ligands for β_1 (Atenolol) and β_2 (ICI 118,551) adrenergic receptors competed with [125I]CYP (300 pM) for β-adrenergic binding sites in cell membrane fractions from the pancreatic carcinoma cell line **Panc-1**. Both competition curves represent nonlinear regression isotherm for two classes of binding sites. Both ligands bound high and low affinity sites. Ligand concentrations that blocked 50% of CYP binding (EC_{50}) for each competing ligand for each class of binding sites were as follows: Atenolol EC_{50}-1: 81.5 nM, EC_{50} -2: 2.2 mM; ICI 118,551 EC_{50}-1: 832 nM, EC_{50}-2: 0.41 mM. The R_2 value for the ICI 118,551 curve was 0.97. The R_2 value for the atenolol curve was 0.94.

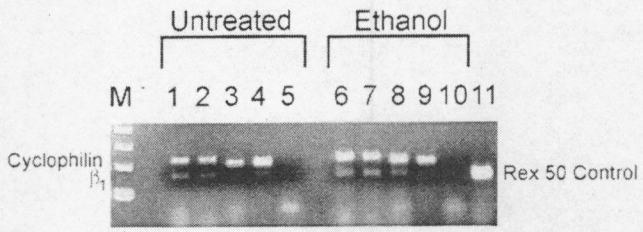

Figure 12: Results of RTPCR for β_1 in **fetal hamster pancreas**. The β_1 primer pair produces a 159 bp product. Cyclophilin is a 216 bp. fragment. Lanes 1,2 and 6,7 represent β_1 primer. Lanes 3,8 represent β_1 primer and cyclophilin control. Lanes 4,9 represent cyclophilin alone. Lanes 5,10 represent negative control. Lane 11 represents positive β_1 control.

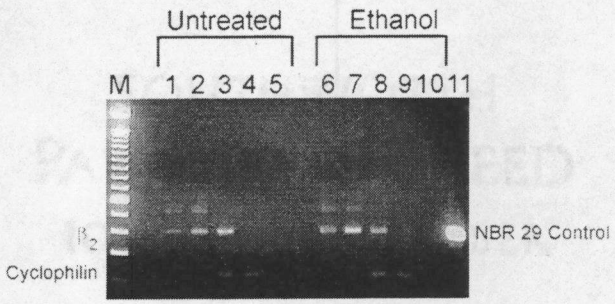

Figure 13: Results of RTPCR for β_2 in **fetal hamster pancreas**. The β_2 primer pair produces a 401 bp product. Cyclophilin is a 216 bp. fragment. Lanes 1,2 and 6,7 represent β_2 primer. Lanes 3,8 represent β_2 primer and cyclophilin control. Lanes 4,9 represent cyclophilin alone. Lanes 5,10 represent negative control. Lane 11 represents positive β_2 control.

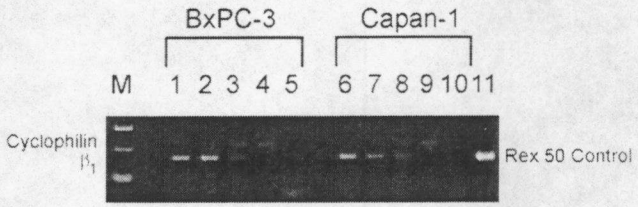

Figure 14: Results of RTPCR for β_1 in human pancreatic carcinoma cell lines **BxPC-3** and **Capan-1**. The β_1 primer pair produces a 159 bp product. Cyclophilin is a 216 bp. fragment. Lanes 1,2 and 6,7 represent β_1 primer. Lanes 3,8 represent β_1 primer and cyclophilin control. Lanes 4,9 represent cyclophilin alone. Lanes 5,10 represent negative control. Lane 11 represents positive β_1 control.

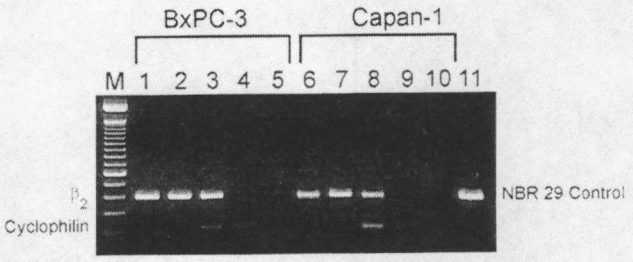

Figure 15: Results of RTPCR for β_2 in human pancreatic carcinoma cell lines **BxPC-3** and **Capan-1**. The β_2 primer pair produces a 401 bp product. Cyclophilin is a 216 bp. fragment. Lanes 1,2 and 6,7 represent β_2 primer. Lanes 3,8 represent β_2 primer and cyclophilin control. Lanes 4,9 represent cyclophilin alone. Lanes 5,10 represent negative control. Lane 11 represents positive β_2 control.

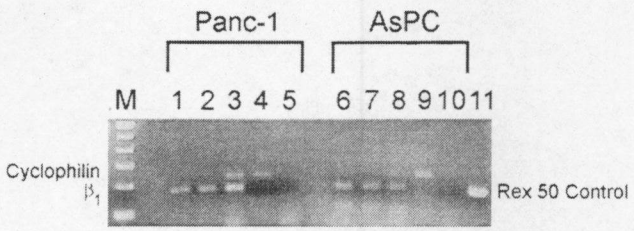

Figure 16: Results of RTPCR for β_1 in human pancreatic carcinoma cell lines **Panc-1** and **AsPC**. The β_1 primer pair produces a 159 bp product. Cyclophilin is a 216 bp fragment. Lanes 1,2 and 6,7 represent β_1 primer. Lanes 3,8 represent β_1 primer and cyclophilin control. Lanes 4,9 represent cyclophilin alone. Lanes 5,10 represent negative control. Lane 11 represents positive β_1 control.

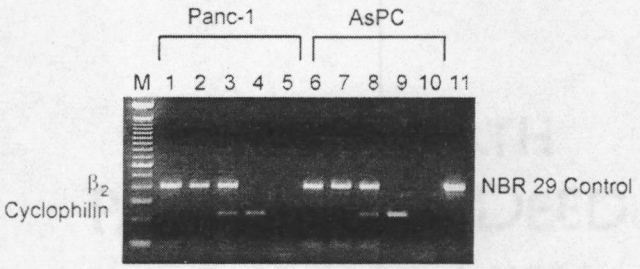

Figure 17: Results of RTPCR for β_2 in human pancreatic carcinoma cell lines **Panc-1** and **AsPC**. The β_2 primer pair produces a 401 bp product. Cyclophilin is a 216 bp. fragment. Lanes 1,2 and 6,7 represent β_2 primer. Lanes 3,8 represent β_2 primer and cyclophilin control. Lanes 4,9 represent cyclophilin alone. Lanes 5,10 represent negative control. Lane 11 represents positive β_2 control.

```
File1: REX50B1F
Mode: Normal                1 -     160
File2: BETA1
Mode: Normal              700 -     900
Matching Percentage (Total Window: 74%, Alignment Window: 100%)

  -46  .............................................AAC        3
                                                    |||
  700  AGGCGCGCCGCTGCTACAACGACCCCAAGTGCTGCGACTTCGTCACCAAC      749

    4  CGGGCCTACGCCATCGCCTCGTCCGTAGTCTCCTTCTACGTGCCCCTGTG       53
       ||||||||||||||||||||||||||||||||||||||||||||||||||
  750  CGGGCCTACGCCATCGCCTCGTCCGTAGTCTCCTTCTACGTGCCCCTGTG      799

   54  CATCATGGCCTTCGTGTACCTGCGGGTGTTCCGCGAGGCCCAGAAGCAGG      103
       ||||||||||||||||||||||||||||||||||||||||||||||||||
  800  CATCATGGCCTTCGTGTACCTGCGGGTGTTCCGCGAGGCCCAGAAGCAGG      849

  104  TGAAGAAGATCGACAGCTGCGAGCGCCGTTTCCTCGGCGGCCCAGCGCGG      153
       ||||||||||||||||||||||||||||||||||||||||||||||||||
  850  TGAAGAAGATCGACAGCTGCGAGCGCCGTTTCCTCGGCGGCCCAGCGCGG      899

  154  CCGCCCT.....................................          203
       |
  900  C...........................................          949
```

Figure 18: Sequence homology matching between the **Rex50** (Chinese hamster ovarian cell line transfected with human β_1-adrenergic receptor gene) RTPCR product and β_1-adrenergic receptor gene sequence. Results show 100% matching within the alignment window.

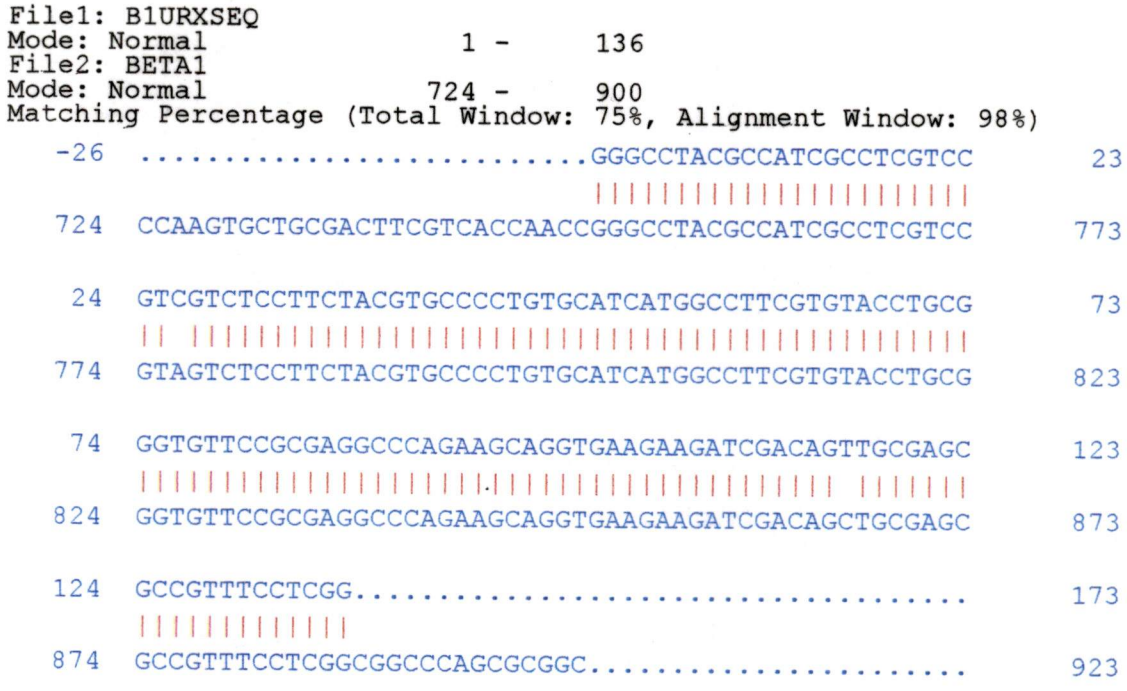

Figure 19: Sequence homology matching between the **untreated fetal pancreas** RTPCR product and β_1-adrenergic receptor gene sequence. Results show 98% matching within the alignment window.

```
File1: B1ETOH
Mode: Normal                1 -     136
File2: BETA1
Mode: Normal              724 -     900
Matching Percentage (Total Window: 75%, Alignment Window: 98%)
   -26  ..........................GGGCCTACGCCATCGCCTCGTCC       23
                                  ||||||||||||||||||||||||
   724  CCAAGTGCTGCGACTTCGTCACCAACCGGGCCTACGCCATCGCCTCGTCC      773

    24  GTCGTCTCCTTCTACGTGCCCCTGTGCATCATGGCCTTCGTGTACCTGCG       73
        || ||||||||||||||||||||||||||||||||||||||||||||||||
   774  GTAGTCTCCTTCTACGTGCCCCTGTGCATCATGGCCTTCGTGTACCTGCG      823

    74  GGTGTTCCGCGAGGCCCAGAAGCAGGTGAAGAAGATCGACAGTTGCGAGC      123
        |||||||||||||||||||||||||||||||||||||||| |||||||
   824  GGTGTTCCGCGAGGCCCAGAAGCAGGTGAAGAAGATCGACAGCTGCGAGC      873

   124  GCCGTTTCCTCGG.....................................      173
        ||||||||||||
   874  GCCGTTTCCTCGGCGGCCCAGCGCGGC.......................      923
```

Figure 20: Sequence homology matching between the **ethanol treated fetal pancreas** RTPCR product and β_1-adrenergic receptor gene sequence. Results show 98% matching within the alignment window.

```
File1: B1BXPC
Mode: Normal                1 -     135
File2: BETA1
Mode: Normal              724 -     900
Matching Percentage (Total Window: 75%, Alignment Window: 98%)
  -27  ..........................GGCCTACGCCATCGCCTCGTCC       22
                                 ||||||||||||||||||||||
  724  CCAAGTGCTGCGACTTCGTCACCAACCGGGCCTACGCCATCGCCTCGTCC      773

   23  GTAGTCTCCTTCTACGTGCCCCTGTGCATCATGGCCTTCGTGTACCTGCG       72
       ||||||||||||||||||||||||||||||||||||||||||||||||||
  774  GTAGTCTCCTTCTACGTGCCCCTGTGCATCATGGCCTTCGTGTACCTGCG      823

   73  GGTGTTCCGCGAGGCCCAGAAGCAGGTGAAGAAGATCGACAGGTGCCAGC      122
       |||||||||||||||||||||||||||||||||||||||| ||| |||
  824  GGTGTTCCGCGAGGCCCAGAAGCAGGTGAAGAAGATCGACAGCTGCGAGC      873

  123  GCCGTTTCCTCGG.....................................      172
       ||||||||||||
  874  GCCGTTTCCTCGGCGGCCCAGCGCGGC.......................      923
```

Figure 21: Sequence homology matching between the human pancreatic carcinoma cell line **BxPC-3** RTPCR product and β_1-adrenergic receptor gene sequence. Results show 98% matching within the alignment window.

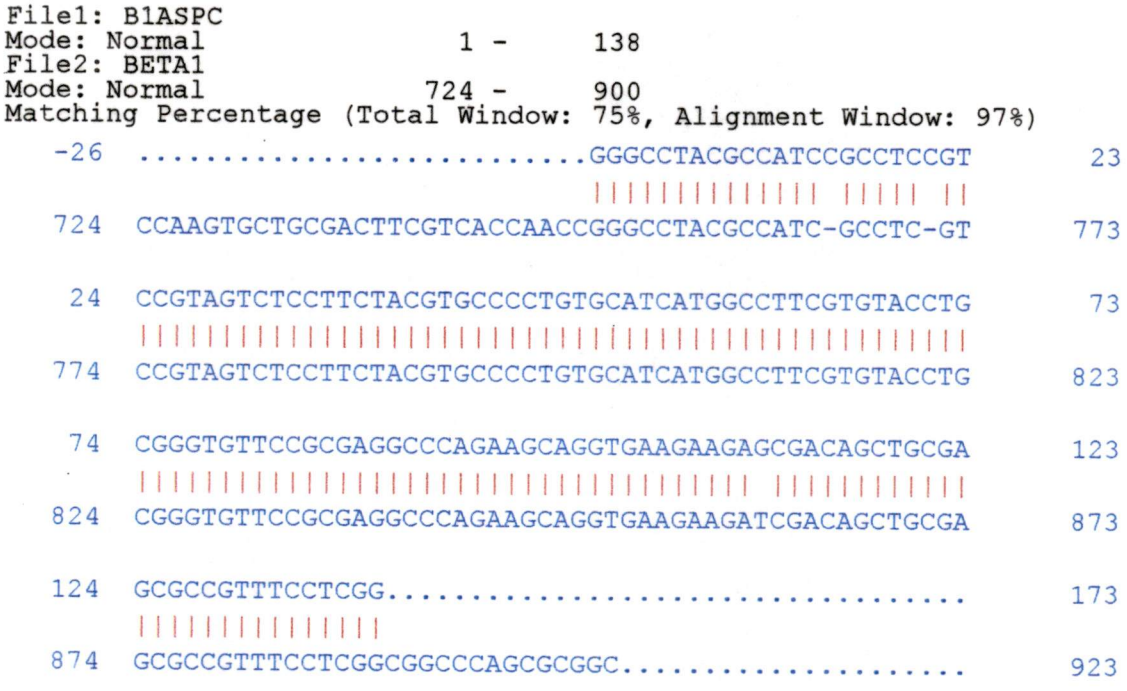

Figure 22: Sequence homology matching between the human pancreatic carcinoma cell line **AsPC** RTPCR product and β_1-adrenergic receptor gene sequence. Results show 97% matching within the alignment window.

Figure 23: Sequence homology matching between the human pancreatic carcinoma cell line **Capan-1** RTPCR product and β_1-adrenergic receptor gene sequence. Results show 98% matching within the alignment window.

```
File1: B1PANC
Mode: Normal                1 -     136
File2: BETA1
Mode: Normal              724 -     900
Matching Percentage (Total Window: 76%, Alignment Window: 100%)
   -26  ..........................GGGCCTACGCCATCGCCTCGTCC       23
                                  |||||||||||||||||||||||
   724  CCAAGTGCTGCGACTTCGTCACCAACCGGGCCTACGCCATCGCCTCGTCC      773

    24  GTAGTCTCCTTCTACGTGCCCCTGTGCATCATGGCCTTCGTGTACCTGCG       73
        ||||||||||||||||||||||||||||||||||||||||||||||||||
   774  GTAGTCTCCTTCTACGTGCCCCTGTGCATCATGGCCTTCGTGTACCTGCG      823

    74  GGTGTTCCGCGAGGCCCAGAAGCAGGTGAAGAAGATCGACAGCTGCGAGC      123
        ||||||||||||||||||||||||||||||||||||||||||||||||||
   824  GGTGTTCCGCGAGGCCCAGAAGCAGGTGAAGAAGATCGACAGCTGCGAGC      873

   124  GCCGTTTCCTCGG.....................................      173
        |||||||||||||
   874  GCCGTTTCCTCGGCGGCCCAGCGCGGC.......................      923
```

Figure 24: Sequence homology matching between the human pancreatic carcinoma cell line **Panc-1** RTPCR product and β_1-adrenergic receptor gene sequence. Results show 100% matching within the alignment window.

```
File1: NBRB2F
Mode: Normal                1 -     203
File2: BETA2
Mode: Normal             1660 -    2000
Matching Percentage (Total Window: 59%, Alignment Window: 99%)
  -17  ..................GTGTGGGTGGTGGGCATGGGCATCGTCATGTC       32
                          ||||||||||||||||||||||||||||||||
 1660  ACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGTCATGTC     1709

   33  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA       82
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1710  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA     1759

   83  TTGCCAAGTTCGAGCGTCTGGAGACGGTCACCAACTACTTCATCACTTCA      132
       |||||||||||||||||||| ||||||||||||||||||||||||||||||
 1760  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA     1809

  133  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC      182
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1810  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC     1859

  183  CGCCCATATTCTTATGAAAAT.............................      232
       ||||||||||||||||||||
 1860  CGCCCATATTCTTATGAAAATGTGGACTTTTGGCAACTTCTGGTGCGAGT     1909
```

Figure 25: Sequence homology matching between **NBR29** (Chinese hamster ovarian cell line transfected with human β_2-adrenergic receptor gene) RTPCR product and β_2-adrenergic receptor gene sequence. Results show 97% matching within the alignment window.

```
File1: B2UNRX
Mode: Normal                1 -     185
File2: BETA2
Mode: Normal             1660 -    1900
Matching Percentage (Total Window: 74%, Alignment Window: 95%)

  -31  ................................CAT---C--CGTCATTGT       18
                                       |||   |  ||||||  ||
 1660  ACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGTCAT-GT     1709

   19  CTCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCC       68
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1710  CTCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCC     1759

   69  ATTGCCAAGTTCGAGCGTCTGCAGACGGACACCAACTACTACATCACTTC       118
       |||||||||||||||||||||||||| |||||||||| |||||||||
 1760  ATTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTC     1809

  119  ACTGGCCTGTGCTGATCTGGTCATGGGCCTAGCAGTGGTGCCCTTTGGGG       168
       |||||||||||||||||||||||||| ||||||||||||||||||
 1810  ACTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGG     1859

  169  CCGCCCATATTCTTATGAAAAT.........................       218
       ||||||||||||||||||||
 1860  CCGCCCATATTCTTATGAAAATGTGGACTTTTGGCAACTTCT........     1909
```

Figure 26: Sequence homology matching between the **untreated fetal pancreas** RTPCR product and human β_2-adrenergic receptor gene sequence. Results show 95% matching within the alignment window.

```
File1: B2ETOH
Mode: Normal                1 -     171
File2: BETA2
Mode: Normal             1660 -    1900
Matching Percentage (Total Window: 69%, Alignment Window: 97%)

  -37  ....................................CATCGTCATGTC       12
                                           ||||||||||||
 1660  ACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGTCATGTC    1709

   13  TCTCATCGTCCTGGCCATCGTGTTTGGGCAATGTGCTGGTCATCACAGCC      62
       |||||||||||||||||||||||||| ||||||||||||||||||||||
 1710  TCTCATCGTCCTGGCCATCGTGTTTGG-CAATGTGCTGGTCATCACAGCC    1759

   63  ATTGCCAAGTTCGAGCGTCTGCAGACGGACACCAACTACTACATCACTTC      112
       |||||||||||||||||||||||||| |||||||||| |||||||||
 1760  ATTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTC    1809

  113  ACTGGCCTGTGCTGATCTGGTCATGGGCCTAGCAGTGGTGCCCTTTGGGG     162
       |||||||||||||||||||||||||| ||||||||||||||||||
 1810  ACTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGG    1859

  163  CCGCCCATA.....................................      212
       |||||||||
 1860  CCGCCCATATTCTTATGAAAATGTGGACTTTTGGCAACTTCT........    1909
```

Figure 27: Sequence homology matching between the **ethanol treated fetal pancreas** RTPCR product and human β_2-adrenergic receptor gene sequence. Results show 97% matching within the alignment window.

```
File1: BXPCB2.SEQ
Mode: Normal                1 -     350
File2: BETA2
Mode: Normal             1660 -    2000
Matching Percentage (Total Window: 87%, Alignment Window: 97%)

  -17  ..................GTGTGGGTGGTGGGCATGGGCATCGTCATGTC       32
                          ||||||||||||||||||||||||||||||||
 1660  ACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGTCATGTC     1709

   33  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA       82
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1710  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA     1759

   83  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA      132
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1760  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA     1809

  133  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC      182
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1810  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC     1859

  183  CGCCCATATTCT-ATGAAAAATGTGGACTTTTGGCAACTTCTGGTGCGAG      232
       |||||||||||| ||||||| ||||||||||||||||||||||||||||||
 1860  CGCCCATATTCTTATGAAAA-TGTGGACTTTTGGCAACTTCTGGTGCGAG     1909

  233  TTTTGGACTTCCATTTGATGTGCTGTGCGTCACGGCCAGCATTGAAGACC      282
       ||||||||||||||| |||||||||||||||||||||||||||||| ||||
 1910  TTTTGGACTTCCATT-GATGTGCTGTGCGTCACGGCCAGCATTGA-GACC     1959

  283  CTGTGCGTTGATCGCAGTGGGATCGCTACTTTTGCCATTACTTCACCTTT      332
       |||||||| |||||||||||| |||||||||||| ||||||||||||||
 1960  CTGTGCGT-GATCGCAGTGG-ATCGCTACTTT-GCCATTACTTCACC...     2009
```

Figure 28: Sequence homology matching between the human pancreatic adenocarcinoma **BxPC-3** cell line RTPCR product and human β_2-adrenergic receptor gene sequence. Results show 97% matching within the alignment window.

```
File1: ASPCB2.SEQ
Mode: Normal                1 -     380
File2: BETA2
Mode: Normal             1660 -    2000
Matching Percentage (Total Window: 79%, Alignment Window: 95%)

   -17  ..................TTTTGGGTGGTGGGCATGGGCATCGTCATGTC       32
                           | ||||||||||||||||||||||||||||||
  1660  ACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGTCATGTC     1709

    33  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA       82
        ||||||||||||||||||||||||||||||||||||||||||||||||||
  1710  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA     1759

    83  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA      132
        ||||||||||||||||||||||||||||||||||||||||||||||||||
  1760  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA     1809

   133  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC      182
        ||||||||||||||||||||||||||||||||||||||||||||||||||
  1810  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC     1859

   183  CGCCCATATTCT-ATGNAAAATGTGGACTTTTGGCAACTTCTGGTGNCNA      232
        |||||||||||| ||| ||| |||||||||||||||||||||||    |
  1860  CGCCCATATTCTTATGAAAA-TGTGGACTTTTGGCAACTTCTGGTGC-GA     1909

   233  GTTTTGGACTTCCATTTGATGTGCTGTGCGTCACGGCCAGCATTTGAAGA      282
        ||||||||||||||| |||||||||||||||||||||||||| || ||
  1910  GTTTTGGACTTCCATT-GATGTGCTGTGCGTCACGGCCAGCATT-GA-GA     1959

   283  CCCTGTGCGTTGATCGCAGTGGGATCGCTACTTTTGCCATTACTTCACCT      332
        |||||||||| |||||||||| |||||||||| |||||||||||||
  1960  CCCTGTGCGT-GATCGCAGTGG-ATCGCTACTTT-GCCATTACTTCACC.     2009
```

Figure 29: Sequence homology matching between the human pancreatic adenocarcinoma **AsPC** cell line RTPCR product and human β_2-adrenergic receptor gene sequence. Results show 95% matching within the alignment window.

```
File1: CAPANB2.SEQ
Mode: Normal                1 -     350
File2: BETA2
Mode: Normal             1660 -    2000
Matching Percentage (Total Window: 87%, Alignment Window: 100%)

   -17  ..................GTGTGGGTGGTGGGCATGGGCATCGTCATGTC       32
                           ||||||||||||||||||||||||||||||||
  1660  ACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGTCATGTC     1709

    33  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA       82
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
  1710  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA     1759

    83  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA       132
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
  1760  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA     1809

   133  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC       182
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
  1810  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC     1859

   183  CGCCCATATTCTTATGAAAATGTGGACTTTTGGCAACTTCTGGTGCGAGT       232
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
  1860  CGCCCATATTCTTATGAAAATGTGGACTTTTGGCAACTTCTGGTGCGAGT     1909

   233  TTTGGACTTCCATTGATGTGCTGTGCGTCACGGCCAGCATTGAGACCCTG       282
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
  1910  TTTGGACTTCCATTGATGTGCTGTGCGTCACGGCCAGCATTGAGACCCTG     1959

   283  TGCGTGATCGCAGTGGATCGCTACTTTGCCATTACTTCACCTTTCCAAGT       332
        ||||||||||||||||||||||||||||||||||||
  1960  TGCGTGATCGCAGTGGATCGCTACTTTGCCATTACTTCACC.........     2009
```

Figure 30: Sequence homology matching between the human pancreatic adenocarcinoma **Capan-1** cell line RTPCR product and human β_2-adrenergic receptor gene sequence. Results show 100% matching within the alignment window.

```
File1: PANCB2.SEQ
Mode: Normal                1 -     380
File2: BETA2
Mode: Normal             1660 -    2000
Matching Percentage (Total Window: 80%, Alignment Window: 97%)

  -17  ..................GTGTGGGTGGTGGGCATGGGCATCGTCATGTC      32
                          ||||||||||||||||||||||||||||||||
 1660  ACGCAGCAAAGGGACGAGGTGTGGGTGGTGGGCATGGGCATCGTCATGTC    1709

   33  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA     82
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1710  TCTCATCGTCCTGGCCATCGTGTTTGGCAATGTGCTGGTCATCACAGCCA    1759

   83  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA     132
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1760  TTGCCAAGTTCGAGCGTCTGCAGACGGTCACCAACTACTTCATCACTTCA    1809

  133  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC     182
       ||||||||||||||||||||||||||||||||||||||||||||||||||
 1810  CTGGCCTGTGCTGATCTGGTCATGGGCCTGGCAGTGGTGCCCTTTGGGGC    1859

  183  CGCCCATATTCTTATGAAAAATGTGGACTTTTGGCAACTTCTGGTGCCGA     232
       |||||||||||||||||| |||||||||||||||||||||||||| ||
 1860  CGCCCATATTCTTATGAAAA-TGTGGACTTTTGGCAACTTCTGGTGC-GA    1909

  233  GTTTTGGACTTCCATTTGATGTGCTGTGCGTCACGGCCAGCATTGAAGAC     282
       ||||||||||||||| |||||||||||||||||||||||||||| |||
 1910  GTTTTGGACTTCCATT-GATGTGCTGTGCGTCACGGCCAGCATTGA-GAC    1959

  283  CCTGTGCGTTGATCGCAGTTGGATCGCTATTTTGCCATTACTTCACCTTT     332
       |||||||| |||||||| |||||||| ||||||||||||||||||
 1960  CCTGTGCGT-GATCGCAGT-GGATCGCTACTTTGCCATTACTTCACC...    2009
```

Figure 31: Sequence homology matching between the human pancreatic adenocarcinoma **Panc-1** cell line RTPCR product and human β_2-adrenergic receptor gene sequence. Results show 97% matching within the alignment window.

APPENDIX B

TABLES

Table 1
Assay Concentrations for Competition Studies

Atenolol; ICI 118,551	Norepinehine; Epine	NNK
0	0	0
1 pM	1 pM	1 pM
10 pM	10 pM	10 pM
100 pM	100 pM	100 pM
1 nM	1 nM	1 nM
10 nM	10 nM	10 nM
100 nM	100 nM	100 nM
1 μM	1 μM	1 μM
10 μM	10 μM	10 μM
100 μM	100 μM	100 μM
1 mM	1 mM	1 mM
	10 mM	10 mM
	100 mM	100 mM

Table 2
Saturation Assay
Statistical Comparison of B_{max}

	Parameter	Value
	X	Y
1	Table Analyzed	Data Table-1 Columns A and B
2		
3	Unpaired t test with Welch's correction	
4	P value	0.2118
5	P value summary	ns
6	Are means signif. different? (P < 0.05)	No
7	One- or two-tailed P value?	Two-tailed
8	Welch-corrected t, df	t=1.334 df=10
9		
10	How big is the difference?	
11	Mean ± SEM of column A	233.2 ± 27.52 N=9
12	Mean ± SEM of column B	328.4 ± 65.85 N=9
13	Difference between means	-95.20 ± 71.37
14	95% confidence interval	-63.81 to 254.2
15	R squared	0.1511
16		
17	F test to compare variances	
18	F,DFn, Dfd	5.726, 8, 8
19	P value	0.0117
20	P value summary	*
21	Are variances significantly different?	Yes

Table 2: Statistical comparison of B_{max} (receptor number) derived from the saturation curves in untreated fetal pancreas and ethanol treated pancreas by unpaired t-test with Welch's correction for unequal variances. Calculated p value is 0.2 based on a 95% confidence interval.

PART III

INFLUENCE OF NNK, ETHANOL, AND SUBTYPE SELECTIVE BETA-ADRENERGIC ANTAGONIST ON CELL PROLIFERATION IN SELECTED PANCREATIC CARCINOMA CELL LINES

Chapter 1: Introduction

I. Brief overview

Part III of this dissertation is devoted to addressing the objective as to whether NNK would induce cell proliferation beyond that observed in controls as measured by DNA synthesis in four different pancreatic carcinoma cell lines (BxPC-3, AsPC-1, Capan-1, and Panc-1). In addition, the objective was to determine if there were any differences in DNA synthesis between the treatment groups in cells grown in general (traditional) media conditions versus ethanol media conditions. Finally, the influence of a beta-adrenergic receptor in this process was tested by treatment of the cell lines with subtype specific antagonists. These processes are being tested in pancreatic carcinoma cell lines because at present a good normal ductal epithelial in vitro system is not available.

II. DNA synthesis stimulation by beta-adrenergic receptor ligands

Beta-adrenergic receptors have been detected in pancreatic carcinoma cell lines (Al-Nakkash, 1996), in Ewing's sarcomas (Whitsett, 1983), and in human hepatocellular carcinomas (Belvilacqua, 1991). In the papers on the pancreatic carcinoma cell lines and Ewing's sarcoma, the potential effect on carcinogenesis was not addressed. In studies using pulmonary adenocarcinoma cell lines, β-adrenergic agonists resulted in proliferation of these cell types compared to controls (Park, 1995). Increased numbers of β_2-adrenergic receptors have been found in membrane fractions from tumorized regions of human liver with

hepatocellular carcinomas compared with adjacent healthy regions of those livers. Basal adenylate cyclase activity in the tumor regions was not different from the healthy regions; however, the adenylate cyclase activity significantly increased with isoproterenol (Bevilacqua, 1991). Cell proliferation (i.e. increased numbers of cells or increased DNA synthesis) secondary to exposure to β2-agonists was not studied in this paper. It was postulated that stimulation of cAMP via β_2-adrenergic receptors would result a proliferative response. The proliferative capabilities of cAMP activation via a beta-adrenergic pathway have been several normal cell systems and in murine and human mammary epithelial cells (Dumont, 1989).

III. Effect of ethanol on beta-adrenergic receptors

This topic was discussed in detail in the background section of Chapter 1 and will not be further discussed here.

Chapter 2: Materials and methods

Cell proliferation was determined by measuring DNA synthesis as a function of tritiated thymidine incorporation in BxPC-3, AsPC-1, Capan-1, and Panc-1 pancreatic carcinoma cell lines. DNA synthesis was measured after exposure to increasing concentrations of NNK, no NNK, or NNK with a β_1 or a β_2 adrenergic antagonist. These measurements were taken from cells maintained in general/traditional media and in cells maintained in ethanol containing media.

I. General maintenance of human pancreatic adenocarcinoma cell lines

Human pancreatic carcinoma cell lines (Panc-1, AsPC, BxPC-3, Capan-1) were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 5 % CO_2 in T-75 ml culture flasks in media recommended by ATCC. All cell lines are adherent cells. Panc-1 cells were maintained in DMEM (4.5 g glucose per liter) with 10% FBS. AsPC cells were maintained in RPMI 1640 (without glutamine) with 20% FBS. BxPC-3 cells were maintained in RPMI 1640 (without glutamine) with 10% FBS. Capan-1 cells were maintained in RPMI 1640 (without glutamine) with 15% FBS. Basic media (DMEM and RPMI 1640) was purchased (Biofluids; Rockville, MD); FBS (Biofluids), L-glutamine (Biofluids), and penicillin/streptomycin (were purchased and added separately. Penicillin/streptomycin were added at a concentration of 50,000 units/50,000 mg per 500 ml of media. Glutamine was added at a concentration of 5 ml of 200 mM solution for a final

concentration of 2 mM.

Cells were passed when 90-95% confluent. When passing, media was suctioned from flask. Cells were washed with 6 ml of PBS. PBS was suctioned from the flask. One ml of trypsin was added and the flask was gentlely swirled to allow coating of the cells with the trypsin. Flask was placed on a hot plate at 37°C until cells were fully loosened. Five ml of culture media was then added to the flask. The media was pipetted vigorously to wash cells from flask wall, thoroughly mix the cells, and break up cell clumps. One to 1.5 ml of cell suspension was then added to new T-75 ml culture flask.

II. Procedure for cell counts

Cells were grown to 95% confluency and then trypinized with 1 ml of trypsin. Cells were transferred to a 50 ml sterile centrifuge tube (Fisher Scientific) and 35 mls of tissue culture media was added. Cell counts were obtained using a standard hemocytometer. The cell suspension was mixed by rotating the centrifuge tube. Each chamber was filled with 11 μl of the cell suspension. Cells with the 4 corner and central squares were counted each chamber. This cell number was divided by 10 to obtain average cell count per square. The average cell count per square was multiple by 10^4 to obtain cells/ml. Cells/ml was converted to cells/μl. (Standard procedure for cell counting is in the Sigma Biochemicals and Reagents catalog, page 1844-45 of 1998.)

III. Preliminary cell proliferation assay preparation

Prior to conducting the proliferation assays, cell count density to be used was

determined for each cell line. Increments of cell counts ranging from 5,000 to 50,000 cells were seeded into standard 96 well cell culture plates (View Plate-96; Packard; Meriden, CT) with 200 μl of media. The plates were placed in a standard tissue culture incubator at 37°C. The plates were examined at 12, 24, and 48 hours. The cell density was approximated for each cell count number seeded by estimating the percent of the well floor covered by cells. The cell count that yielded a density that gave approximately 50 % cover at 24 hours and near confluency by 48 hours were used for the proliferation assays. These values were 10,000 cells per well for the BxPC-3 cell line and 15,000 cells per well for the AsPC cell line.

To obtain general information on the time period of proliferation of the cell lines, BxPC-3 cells and AsPC cells were seeded into 96 well plates at the cell counts determined from the above procedure. The cells of each cell line were seeded with 200 μl media into 48 wells in each of 6 plates. At 24 hours post seeding, ^{3}H-thymidine (0.5 μCi/ well) was added to each well using a repeat pipetter. The cells were harvested (see procedure below) at 1, 2, 4, 8, 12, and 24 hours. Counts per minute (cpm) were graphed as a function of time. This data was used to determine time points for harvesting cells in the proliferation assays.

IV. Ethanol exposure to pancreatic carcinoma cell lines

For assays measuring stimulation under general media conditions, cell maintenance procedures above under general maintenance were followed. For assays measuring stimulation under ethanol conditions, these cells were maintained in ethanol before the assay.

Cells were grown to 90-95% confluency, then trypsinized and passed. Following

passage, cells were allowed to settle to flask for 24 hours. The media was then removed and 10 ml of fresh media containing 0.08 g/dl pure ethanol was added to the flask. (12 μl in 10 ml media = 0.08 g/dl.) Cells were maintained in ethanol laced media through a second passage. The cells were used for proliferation assays after confluent following the second passage.

V. Proliferation assay procedure

In brief

Cell proliferation assays were performed with all 4 cell lines using a standard tritiated thymidine incorporation procedure in 96 well plates within a total volume (media plus reagents) of 200 μl with a tritiated thymidine concentration of 0.5 μCi. Stimulation with increasing concentrations of NNK (10 pM, 100 pM, 1 nM, 10 nM, 30 nM, 100 nM, and 1 μM) were measured in both cell lines at 4, 8, and/or 24 hours and compared to an untreated group. Stimulation with the same concentrations of NNK after the cell lines were exposed to ethanol for 2 passage periods was measured in both cell lines at 4, 8, and/or 24 hours and compared to untreated control groups derived from ethanol and general media conditions. DNA synthesis was measured with simultaneous exposure to NNK and either a β_1 (atenolol) and β_2 (ICI 118,551) antagonist. Stimulation/ inhibition was measured under these treatment conditions in general media conditions and after the cell lines were exposed to ethanol for 2 passage periods. These measurements were compared to untreated control groups derived (ethanol and general media conditions) and to equimolar NNK treatment

groups.

General procedure

Cells were grown to 95% confluency and then trypsinized and cell counts were determined (see section labeled cell count procedure). Ten thousand (BxPC-3, Panc-1) or fifteen thousand (AsPC, Capan-1) cells were seeded into four 96 well plates. Total volume of cells and media in each well was 200 μl. Cell plates were placed in a standard incubator at 37°C for 24 hours.

Each treatment group in all assays had 6 replicates. Reagents and tritiated thymidine were added to the media and this media solution was added at a volume of 200 μl per well. (Except in the case of antagonist/ NNK treatments, the antagonist was added to the media.) Therefore, each treatment group had a separate media preparation. Amount of media needed was determined by number of wells per treatment for one plate + approximately 50% multiplied by number of plates needed (2 time points=2 plates; 4 time points=4 plates). Dilution concentrations used for each drug treatment was those concentrations that would result in the desired assay concentration as a 10 μl volume. Tridiated thymidine was used at a concentration of 0.5 μCi/ well. As an example, for an NNK treatment with an assay concentration of 10 pM to be measured as 12 replicates for 4 time points required the following:

12 wells ---> 20 wells X 4 plates = 80 wells

80 wells X 200 μl = 16 ml media needed

10 μl of a 200 pM NNK would result in 10 pM NNK in 200μl

(Based on Conc 1Vol 1 = Conc 2 Vol 2)

80 wells X 10 μl of 200 pM NNK = 800 μl of a 200 pM NNK dilution

0.5 μCi/ well X 80 wells = 40 μCi needed; @ 1μCi/ μl = 40 μl needed

16 ml - 800 μl NNK - 40 μl tridium = 15.16 ml media needed.

Dilutions of NNK, ICI 118,551, and atenolol were made in PBS. The starting dilution for ICI 188,551 was made in sterile water. Dilutions were protected from light.

Tissue culture media was warmed and aliquoted into sterile centrifuge tubes. Appropriate volumes of drug treatments and tritiated thymidine were added to the media. In the case of controls, PBS was added at the same volume used of treatment reagents. Media was removed from the seeded plate by flicking the media from the plate unto a towel. Media preparation with treatments and thymidine was added to each well as a volume of 200 μl using a repeat pipetter. To insure mixing of the reagents and thymidine within the media, each time prior to adding the media to the wells, the solution was vigorous pipetted by repeated filling and displacing the fluid from the pipetter into the centrifuge tube. This mixing procedure was followed after addition for every six wells (i.e, when there were 12 replicates, fluid was added to only 6 wells at a time and then re-mixed).

All treatments were added to an entire plate (i.e. one time point) before proceeding to another plate. Plates, immediately following addition of media preparation were replaced to the incubator and the time was recorded for that particular plate (time point). For studies involving the incubation of beta agonist with an antagonist. The antagonist was given in the

media preparation and the NNK was added as a 10 μl volume using a repeat pipetter 10 minutes following the addition of the media preparation. The plate was then placed on an shaker (Orbit Shaker; Lab-Line) and rotated for 1 minute at 100 RPM.

For assays involving ethanol, the cells were seeded in standard media for the initial 24 hour period. Media preparation with reagents or PBS and thymidine contained absolute, 200 proof ethanol (AAPER; Shelbyville, KY) at a concentration of 0.08 g/dl. A separate control group was performed with these assays and used general media with PBS and no reagents.

VI. Procedure for harvesting incorporated tridiated thymidine

The media/reagents were removed from the cells by flicking the fluid from the plate onto a towel. According to a procedure obtained from Packard (Meriden, CT), 25 μl of 0.1N NaOH was added to each well with a repeat pipetter to lyse cells. The plate was rotated for 5 minutes at 150 RPM. Incorporated thymidine was separated from nonincorporated thymidine by vacuum filtration using a microplate harvester (Harvester; model Micromate 196; Packard) of the lysed cells onto a "unbacked" glass fiber filter. ("Backed" indicated the filter was made in such a way that the harvester O rings would not cut the filter into small round filter units.) With filter still in place, each well was flushed 5 times with 300 μl/well of ultrafiltrated water. DNA was adhered to the filter by a final flush with 200 μl/well of 2-propanol (Sigma).

The Harvester apparatus cut the filter into circular discs corresponding to each well. The filter discs were placed into appropriately labeled glass liquid scintillation vials. 3 ml

of counting cocktail (Bio-Safe II; Research Products International Corp.; Mount Prospect, IL) was added to each vial and the vial was gently shaken. Vials were counted on a Packard scintillation counter (model Tri-Carb 2300TR) .

VII. Statistical analysis

Statistical analysis was completed using Prism (Graph Pad Software; San Diego, CA). One-way analysis of variance (ANOVA) followed by a Dunnett's post test analysis to compare treatment groups to the control within an assay. In the assays with subtype selective antagonists, β_1 and β_2 antagonist groups with NNK were compared via one-way ANOVA followed by a Bonferroni post test to compare selected pairs of means.

Chapter 3: Results

Cell proliferation was measured by DNA synthesis as a function of tritiated thymidine incorporation in four pancreatic carcinoma cell lines (BxPC-3, AsPC-1, Capan-1, Panc-1). DNA synthesis was measured under general media conditions and under the influence of ethanol after stimulation with varying concentrations of NNK. In addition, DNA synthesis was measured under both media conditions after simultaneous exposure to NNK and either a β_1 (atenolol) or a β_2 (ICI 118,551) antagonist. The results are displayed in figures as bar graphs showing counts per minute (cpm) at varying concentrations. Results of statistics for each assay are shown as data printouts. All figures and statistical data are located in separate appendices at the end of part III of the dissertation. Figures are located in appendix A. Statistical data are located in appendix B; statistical data are group by cell line and media condition.

An analysis of variance with a Dunnett's post test was used to compare the NNK treated groups to the control group not treated with NNK. Under general media conditions, NNK did not have an enhanced effect on DNA synthesis compared to the control in any of the cell lines. In only one run of one cell line (BxPC-3) was there statistically significant increases ($p<0.05$ and $p<0.01$) in DNA synthesis at the higher concentrations. In several instances, there was statistically significant ($p<0.05$ and $p<0.01$) decreases in DNA synthesis at the higher NNK concentrations compared to the control. This finding was present in both runs in the Capan-1 cell line, one run in the Panc-1 cell line, and both runs in the BxPC-3 at

the 24 hour time point only. Under ethanol media conditions, the findings were similar to those in the general media conditions in that NNK did not have an enhanced effect on DNA synthesis. In only one run of one cell line (Panc-1) was there statistically significant increases ($p<0.05$ and $p<0.01$) in DNA synthesis at the higher concentrations. In three of the cell lines, there was statistically significant decreases in DNA synthesis at the higher NNK concentrations compared to the controls.

Under general or ethanol media conditions, simultaneous treatment with NNK and a beta 1 adrenergic antagonist, atenolol, did not consistently result in any statistically significant increases or decreases in DNA synthesis when compared to equimolar concentrations of NNK or to controls. This was true at both concentrations of atenolol used, 10 nM and 100 μM. However, simultaneous treatment with NNK and a beta 2 adrenergic antagonist, ICI 118,551, did result in statistically significant decreases in DNA synthesis. In all 4 cell lines, the higher concentration of ICI 118,551 (100μM) resulted in consistent, repeatable statistically significant decreases ($p<0.01$ and $p<0.001$) when compared NNK treatment and with controls. This finding was also consistent and repeatable in all 4 cell lines under ethanol conditions.

Chapter 4: Discussion

The use of thymidine incorporation as a measure of DNA synthesis to assess cell proliferation is a well-established procedure and has been used previously in this laboratory in studies involving using lung cancer cell lines (Park, 1995). NNK concentrations used in this section have been used previously in this laboratory and can be potentially be achieved in humans (Jull, 1999). The statistical method of one-way ANOVA with subsequent post tests to compare specific groups is the appropriate method when comparing differences in means from multiple groups (Neter, 1990; Weiss, 1982; Prism manual [GraphPad Software]). The concentration of ethanol used to expose cells was obtained from data collected from a report on driving after drug or alcohol use (www.health.org/drinkreport). The author was trying to achieve a general concentration that would potentially be expected in chronic comsumers of alcohol.

Under general media conditions, NNK did not result in an enhanced proliferative effect in any of the four pancreatic carcinoma cell lines. The differences observed were attributed to normal variability. In addition, ethanol media conditions did not result in enhanced proliferation with NNK treatment. There was an occasional inhibitory effect with the ethanol treatment.

The statistically significant findings were generally reserved for treatment groups exposed simultaneously to NNK and a β_2 antagonist. β_1 antagonist treatment did not affect DNA synthesis. Ethanol did not change this observation. However, exposure to a β_2

antagonist resulted in statistically significant decreases in DNA synthesis in all cell lines. This decrease was observed when compared to groups receiving equimolar concentrations of NNK alone or control groups with no treatment. These results were repeatable. In addition, this effect was also observed in cells maintained in ethanol media conditions. The extent or percentage of decrease was relatively the same as that observed under general media conditions.

Although the antagonists were given simultaneously with NNK, the inhibitory effect on DNA synthesis observed in the presence of the β_2 antagonist was attributed to an effect by the β_2 antagonist. The resultant DNA synthesis was consistently 40% to 60% of that measured in either the corresponding NNK treatment group or in control groups. It is unknown whether there was a synergistic effect between the NNK and the antagonist. This could be tested by measuring DNA synthesis in the presence of the same concentrations of the β_2 antagonist without NNK.

Demonstrating inhibition with a site selective antagonist suggest that the beta-adrenergic receptor may regulate, at least to some extent, the cell cycle and turnover in these cell lines. The fact that the response was seen in the presence of the β_2 antagonist and not the β_1 antagonist may be a reflection of 1 of 2 theories, or both. First, β receptors were shown to be more prominent in number compared to β_1 receptors in all cell lines based on RT-PCR and radioligand binding studies presented in part II of this dissertation. In general, this effect was more prominent and consistent at the higher concentration of the β_2 antagonist used. This observation could be due to relatively few numbers of beta-adrenergic receptors present and the need for a higher concentration to achieve an effect.

Second, the response could be due to β_1 and β_2 adrenergic receptors operating through different signal transduction pathways. Functional activity of receptor-ligand interaction as measured by changes in a specific second messenger was not assessed in these studies. It is not known whether the decreases in DNA synthesis occurred as a result of inhibition of cAMP activation or if other second messenger molecules and signal transduction pathways were used. The G-protein linked cAMP cascade is well-documented route in which β-adrenergic receptors and their ligands exert their effects (Lefkowitz, 1990; Stiles, 1991). In addition, β-adrenergic receptors have been show to activate pathways involving phospholipases (Borda, 1998; Ruan, 1997). There is recent evidence in cardiomyocytes of a β_2-adrenergic receptor coupled to cytosolic phospholipase A2 which triggers the release of arachidonic acid (Pavoine, 1999).

The role of ethanol in these results is unclear. Overall, ethanol did not enhance and of the findings in these cell proliferation/ DNA synthesis studies. In the binding studies, ethanol treatment in vivo did result in an overall increase in beta-adrenergic receptors and appeared to increase the proportion of β_2-adrenergic receptors compared to β_1 -adrenergic receptors. However, the inhibitory effect of the β2 antagonist was not enhanced in these cell lines after exposure to ethanol. It is possible that the conditions did not adequately mimick those created in the in vivo model in terms of concentration exposure.

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APPENDIX A

FIGURES

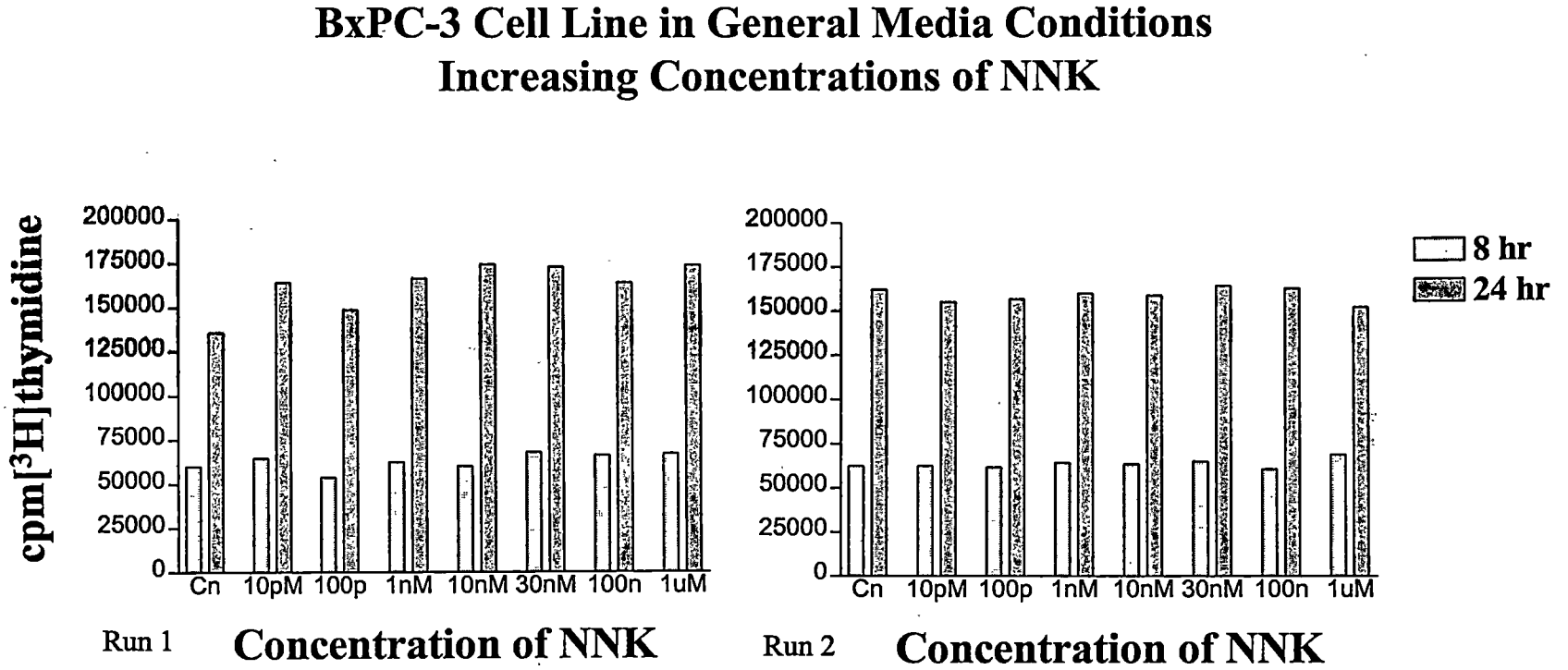

Figure 1: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of increasing concentrations of NNK in general media conditions. Bars represent mean of each treatment group. Graphs shown are represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. At 8 hours in run 1 and 8 and 24 hours in run 2, NNK treatment groups were not statistically significant from the control. At 24 hours in run 1, all concentrations of NNK except 100pM represent a statistically significant increase compared to control; p values <0.05 and <0.01.

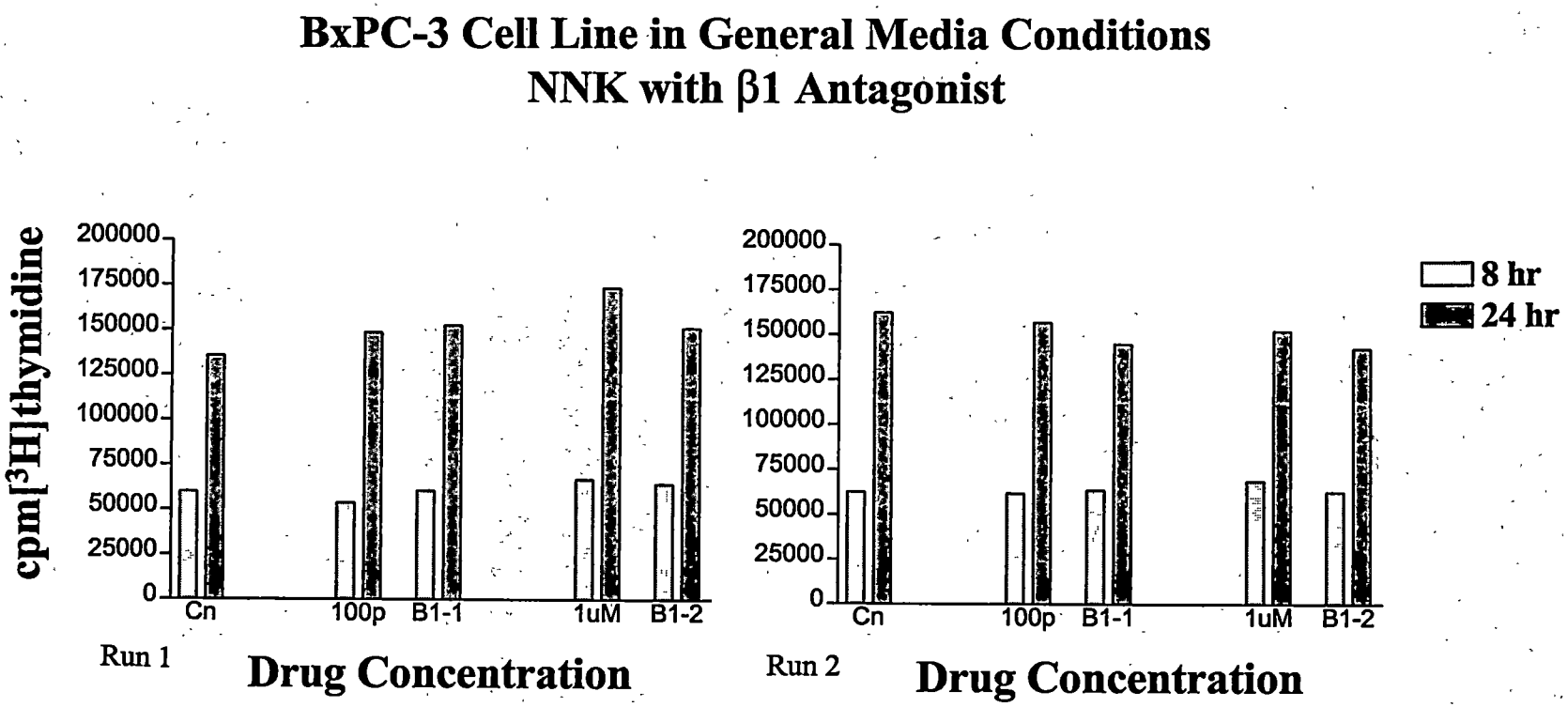

Figure 2: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B1-1=10 nM atenolol + 100 pM NNK. B1-2=100 μM atenolol + 1 μM NNK. The only statistically significant finding is in the higher concentration group in run 2 which resulted in a statistically significant decrease, $p<0.05$ compared to NNK alone.

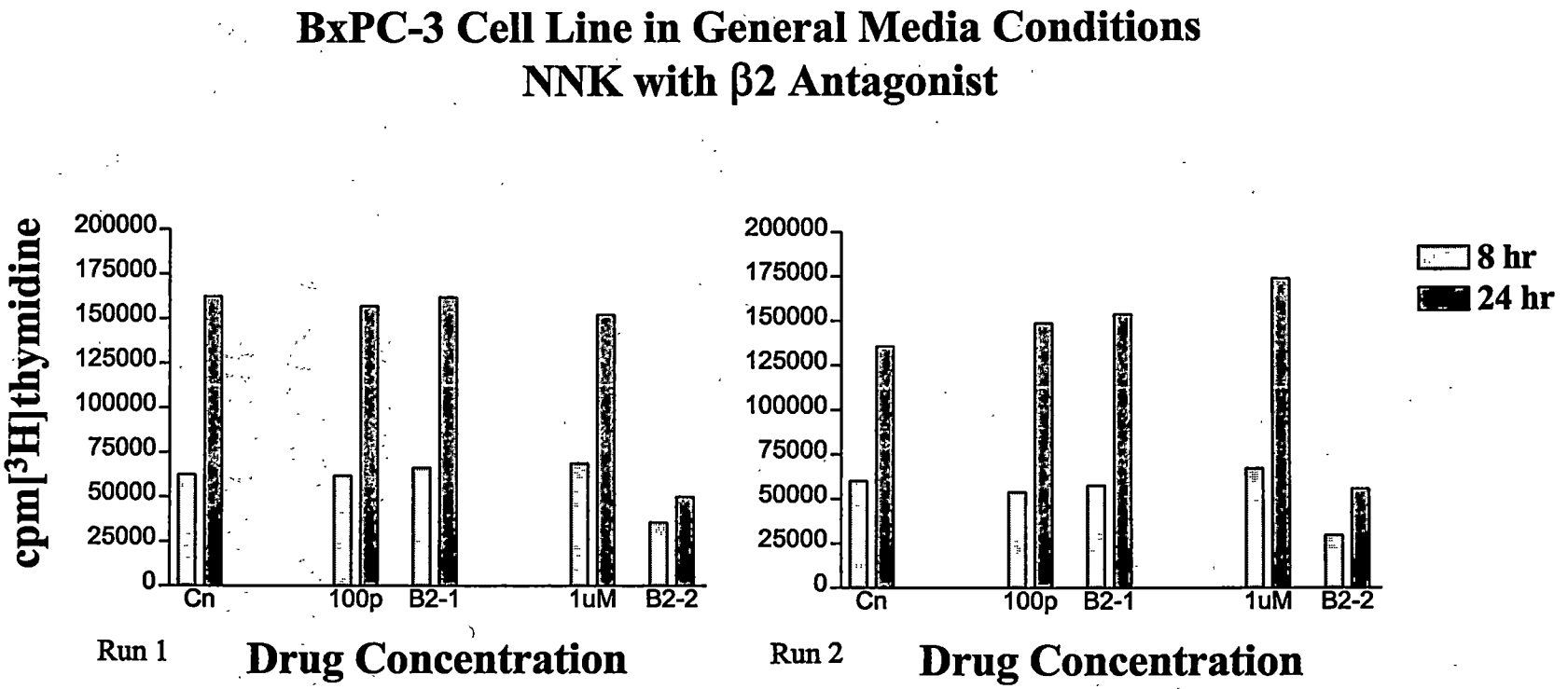

Figure 3: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B2-1=10 nM ICI 118,551 + 100 pM NNK. B2-2=100 μM ICI 118,551 + 1 μM NNK. In both run 1 and run 2, the higher concentration of NNK and the β2 antagonist resulted in a statistically significant decrease compared to NNK alone, $p<0.001$, and compared to control, $p<0.01$.

BxPC-3 Cell Line in Ethanol Media Conditions
Increasing Concentrations of NNK

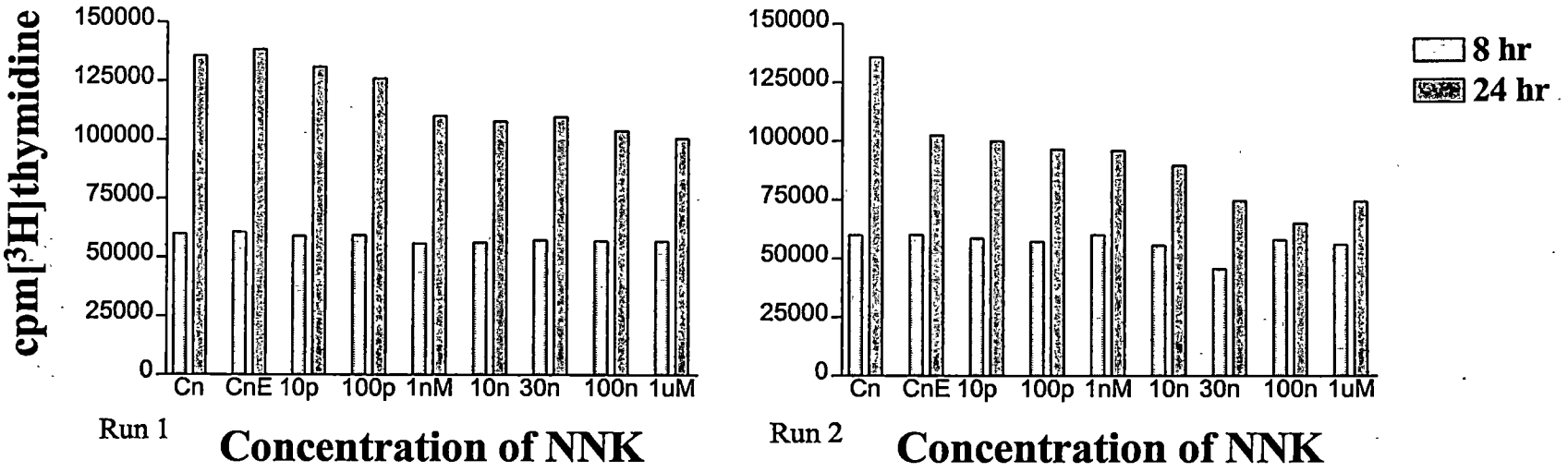

Figure 4: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of increasing concentrations of NNK in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown are represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in presence of PBS and no NNK. There are no significant findings at 8 hours. In run 1 at 24 hours, the 5 highest concentrations of NNK represent statistically significant decreases compared to control or ethanol control, $p<0.01$. In run 2 at 24 hours, all treatment groups and the ethanol control represent statistically significant decreases compared to the control, $p<0.01$. The 3 highest concentrations of NNK represent statistically significant decreases compared to the ethanol control, $p<0.01$.

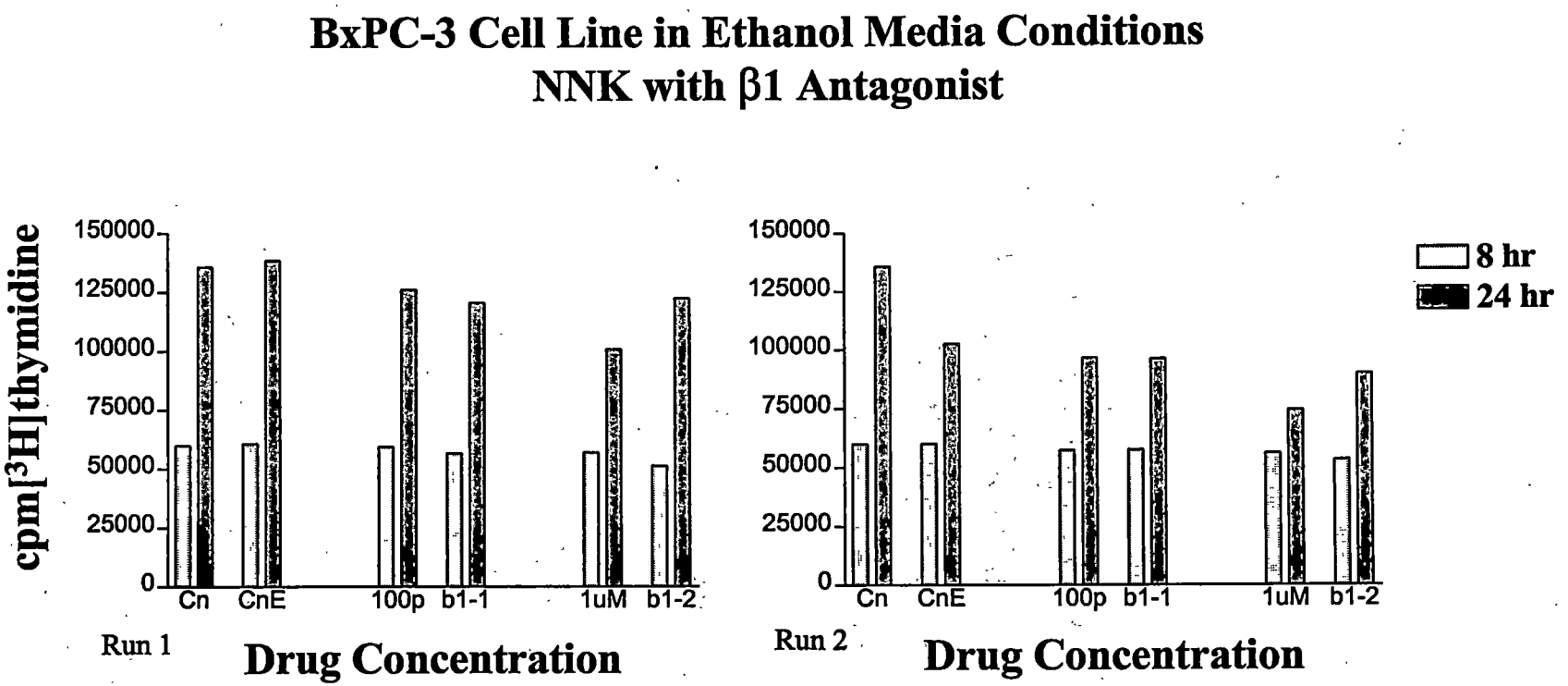

Figure 5: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β1 antagonist in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in the presence of PBS and no NNK. B1-1=10 nM atenolol + 100 pM NNK. B1-2=100 μM atenolol + 1 μM NNK. In run 1 and 2 at 24 hours, the higher NNK and β1 antagonist concentration resulted in statistically significant increase compared to NNK alone, $p<0.01$. In run 2 at 24 hours, the both concentrations resulted in statistically significant decreases compared to the control, $p<0.01$.

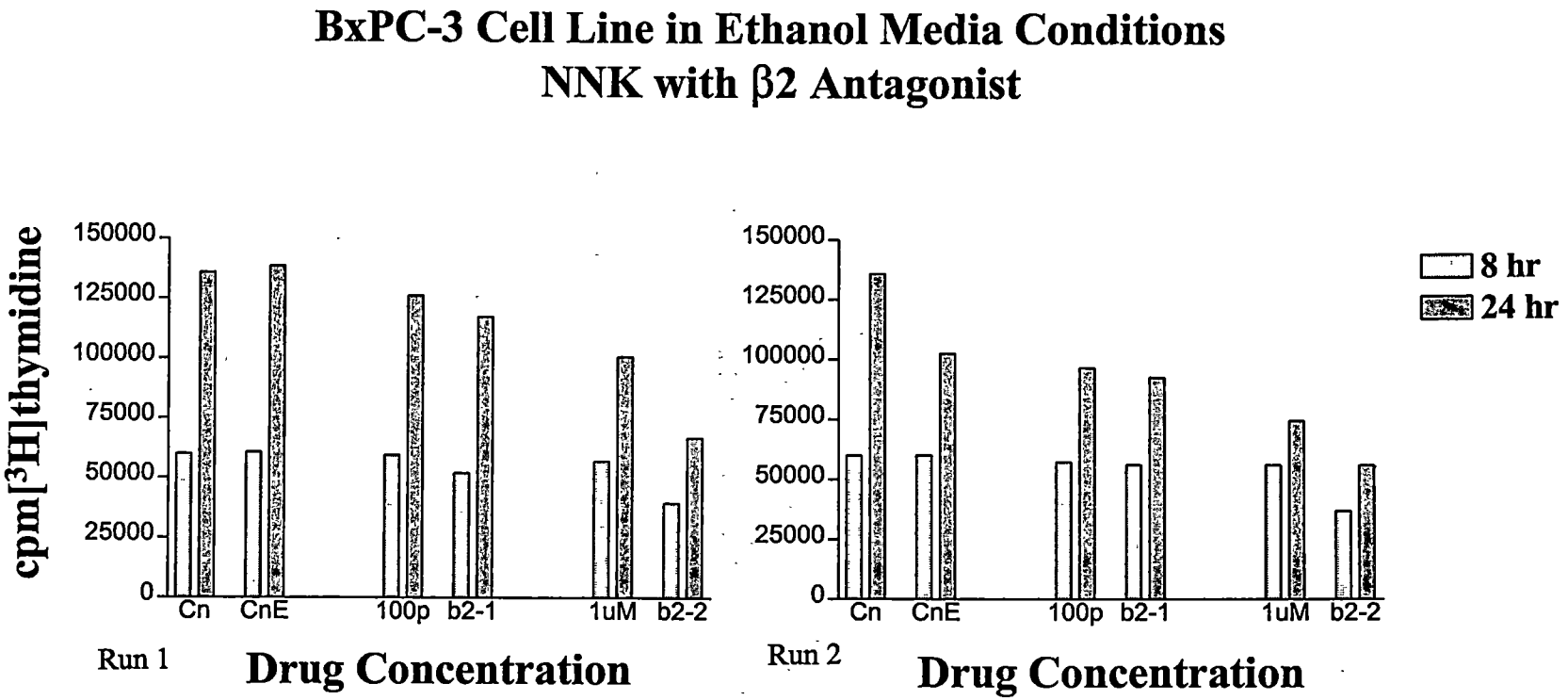

Figure 6: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in presence of PBS and no NNK. B2-1=10 nM ICI 118,551 + 100 pM NNK. B2-2=100 μM ICI 118,551 + 1 μM NNK. In both run 1 and run 2, the higher concentration of NNK and the β2 antagonist resulted in a statistically significant decrease compared to NNK alone, $p<0.001$ (run 1) and $p<0.01$ (run 2), and compared to control and ethanol control, $p<0.01$.

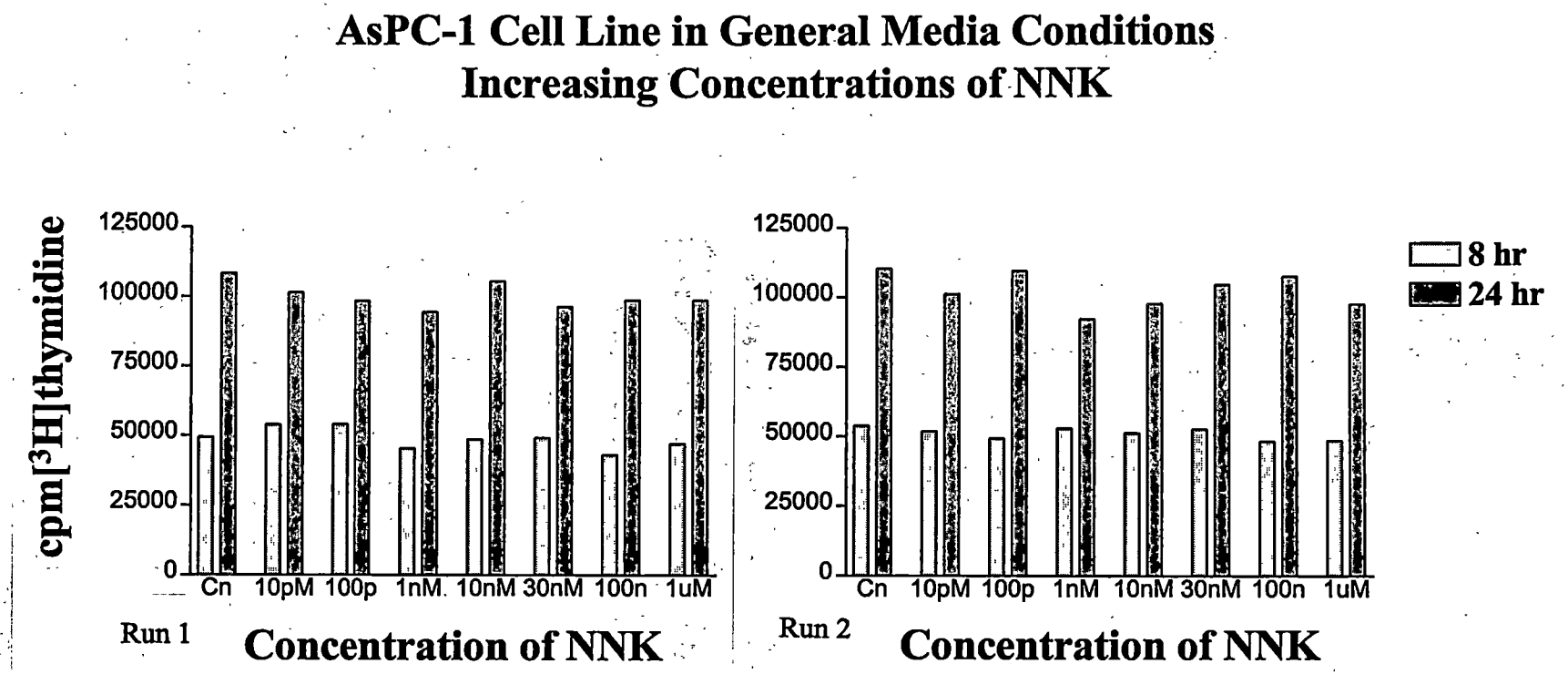

Figure 7: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of increasing concentrations of NNK. in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. There were no statistically significant increases or decreases in the NNK treatment groups compared to the control.

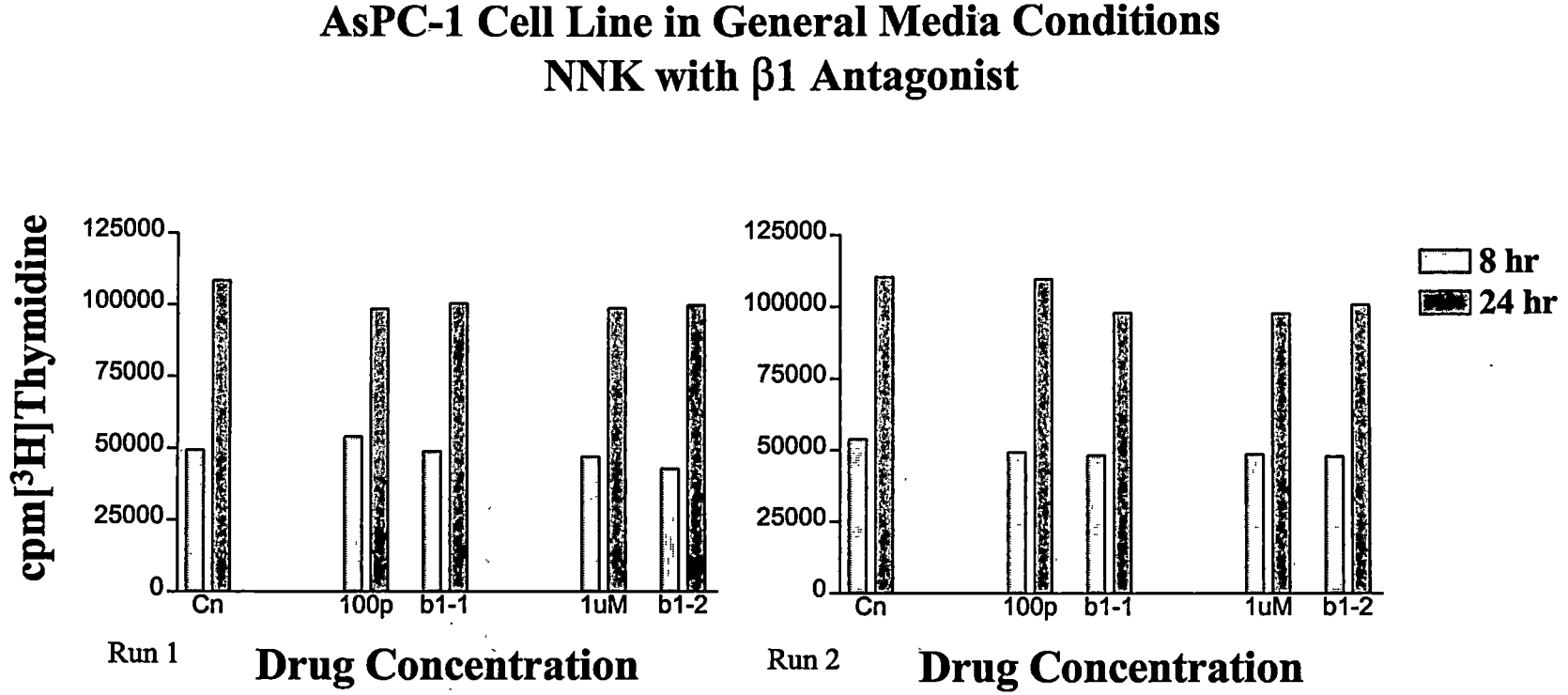

Figure 8: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β1 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B1-1=10 nM atenolol + 100 pM NNK. B1-2=100 μM atenolol + 1 μM NNK. There no were statistically significant findings.

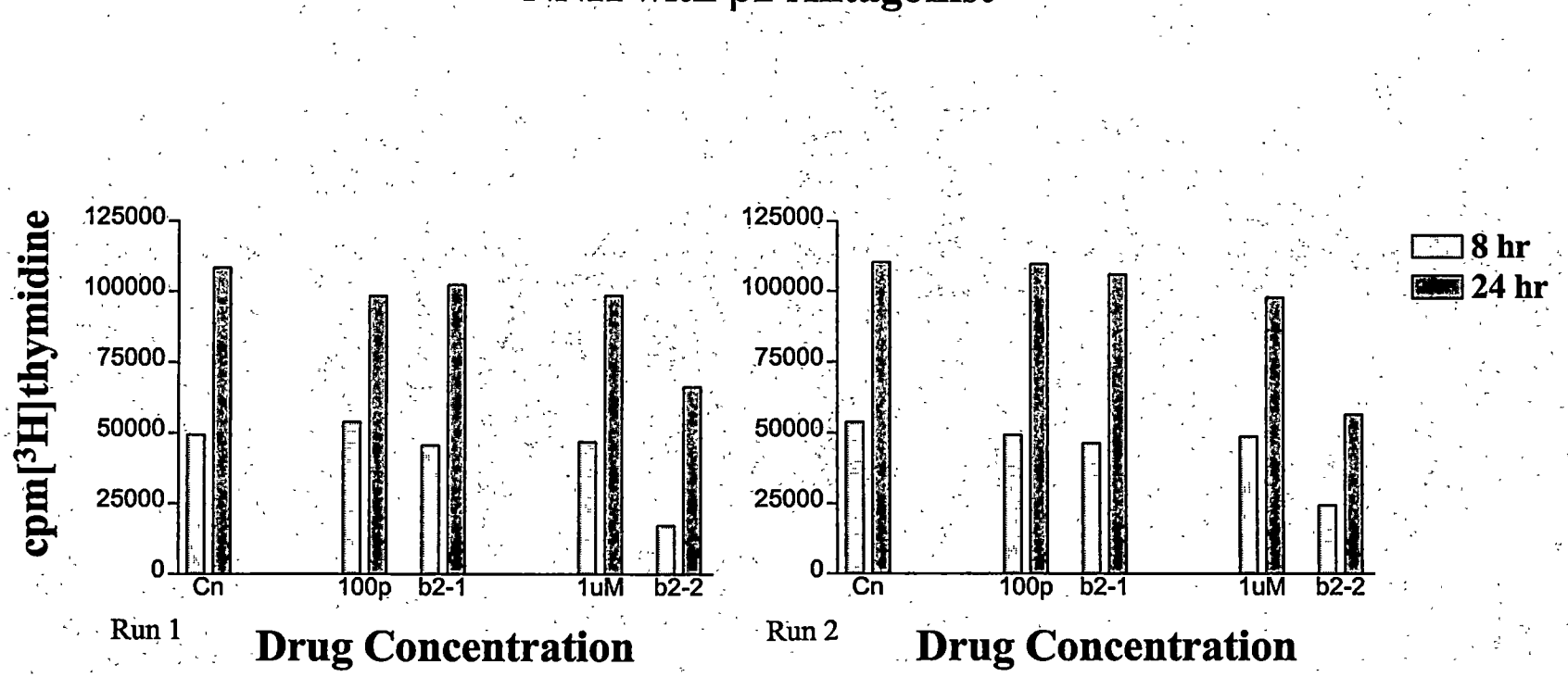

Figure 9: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B2-1=10 nM ICI 118,551 + 100 pM NNK. B2-2=100 μM ICI 118,551 + 1 μM NNK. In both run 1 and run 2, the higher concentration of NNK and the β2 antagonist resulted in a statistically significant decrease, $p<0.001$, compared to NNK alone and compared to the control, $p<0.01$.

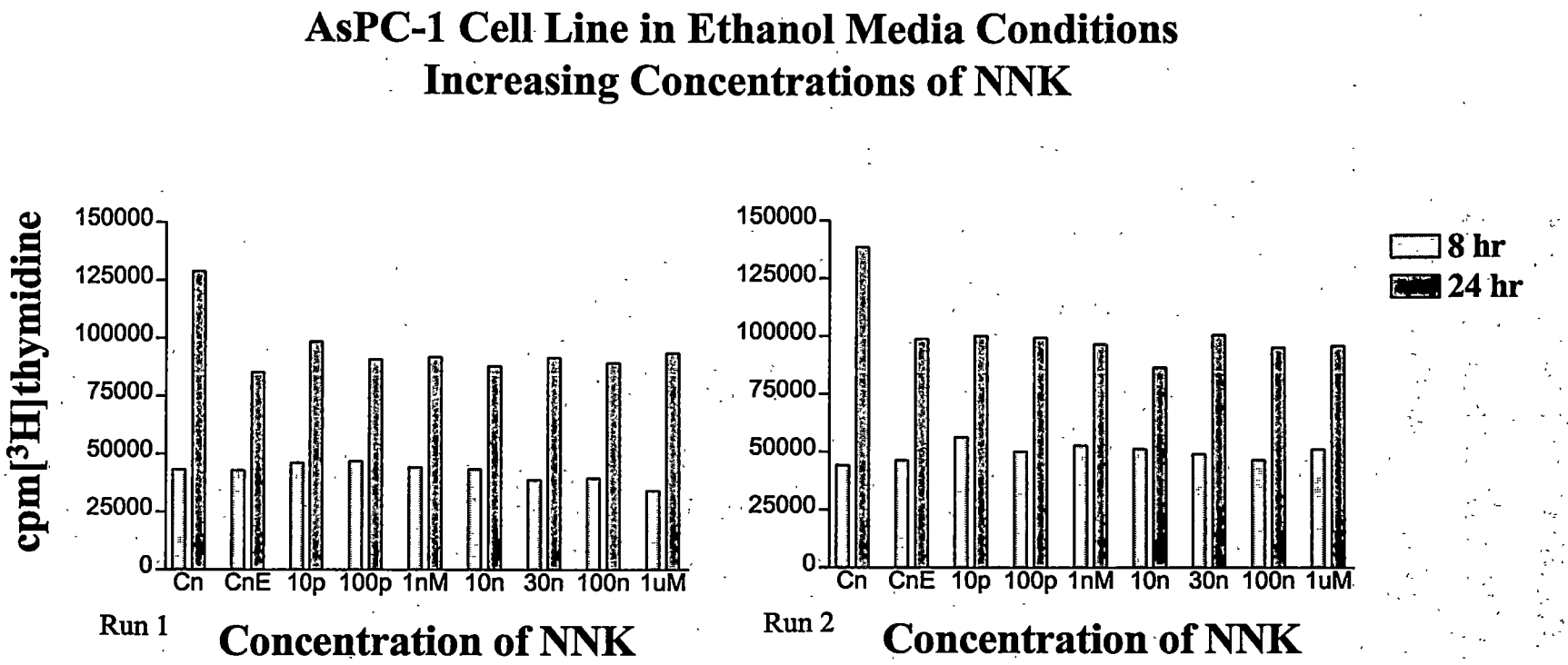

Figure 10: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of increasing concentrations of NNK in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown are represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in presence of PBS and no NNK. In runs 1 and 2 at 24 hours, all treatment groups and the ethanol control represented a statistically significant decrease ($p<0.01$) compared to the control.

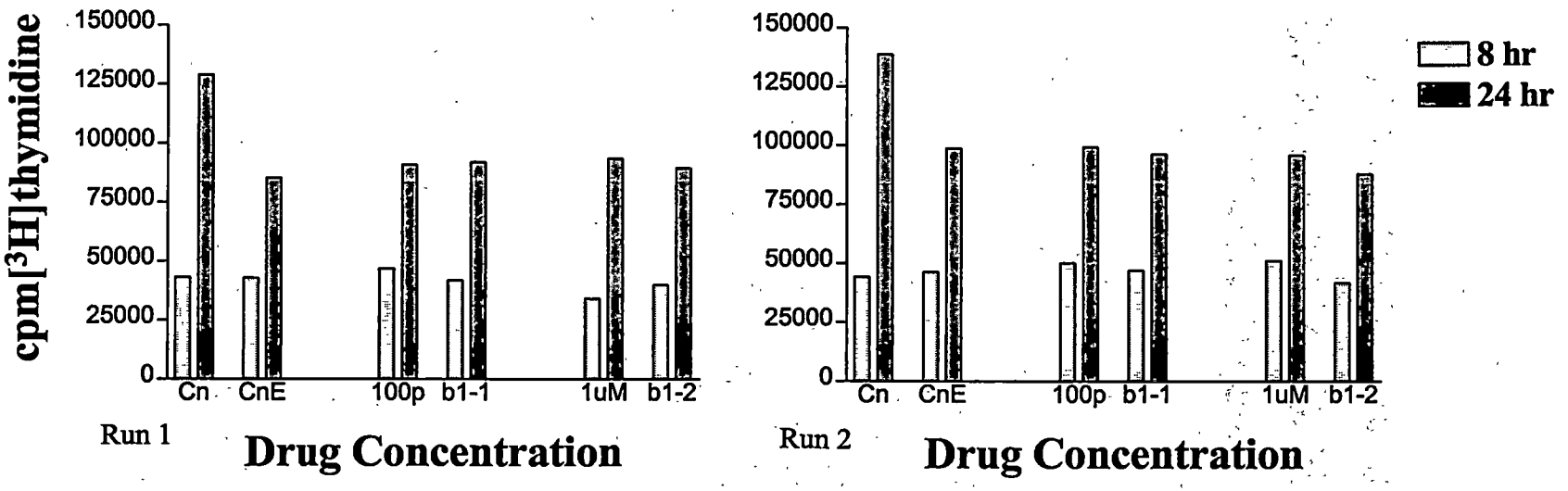

Figure 11: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β1 antagonist in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in the presence of PBS and no NNK. B1-1=10 nM atenolol + 100 pM NNK. B1-2=100 μM atenolol + 1 μM NNK. The only statistical finding was in run 1 at 8 hours, the higher NNK and β1 antagonist concentration resulted a in statistically significant increase compared to NNK alone, $p<0.01$.

AsPC-1 Cell Line in General Media Conditions
NNK with β2 Antagonist

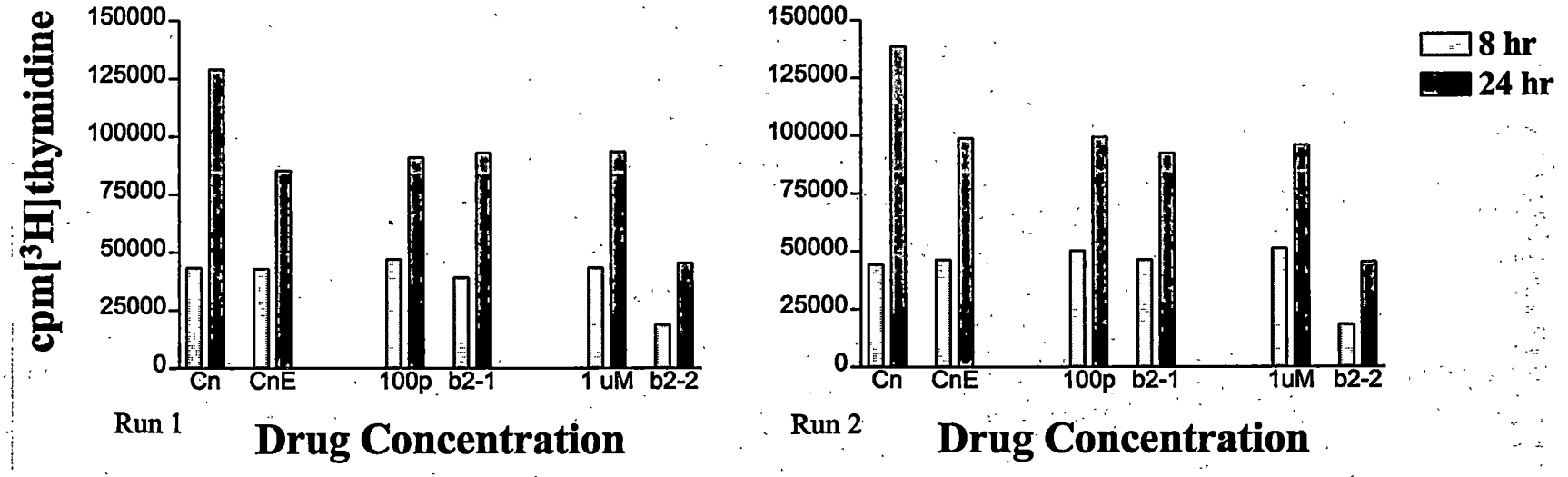

Figure 12: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B2-1=10 nM ICI 118,551 + 100 pM NNK. B2-2=100μM ICI 118,551 + 1 μM NNK. In both run 1 and run 2 at 8 and 24 hours, the higher concentration of NNK and the β2 antagonist resulted in a statistically significant decrease compared to NNK alone, $p<0.001$, and when compared to control and ethanol control, $p<0.01$. In run 1 at 8 hours, the lower concentration resulted in a statistically significant decrease ($p<0.001$) compared to NNK alone.

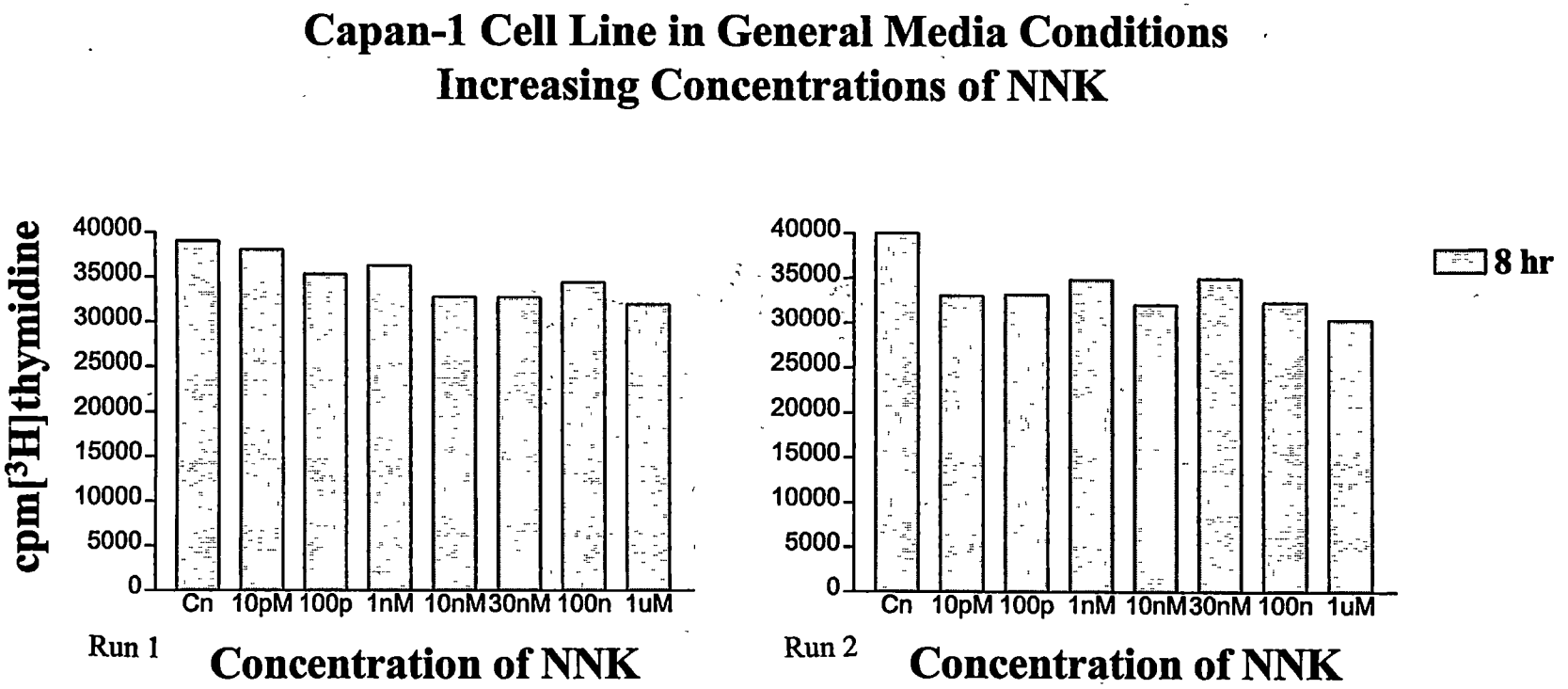

Figure 13: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of increasing concentrations of NNK in general media conditions. Bars represent mean of each treatment group. Graphs shown are represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. In run 1, the 4 highest NNK treatment groups resulted in a statistically significant decrease from the control, $p < 0.05$ and $p < 0.01$. In run 2, 10 pM, 100 pM, 10 nM, 100 nM, 1μM concentration resulted in a statistically significant decrease from the control, $p < 0.05$ and $p < 0.01$.

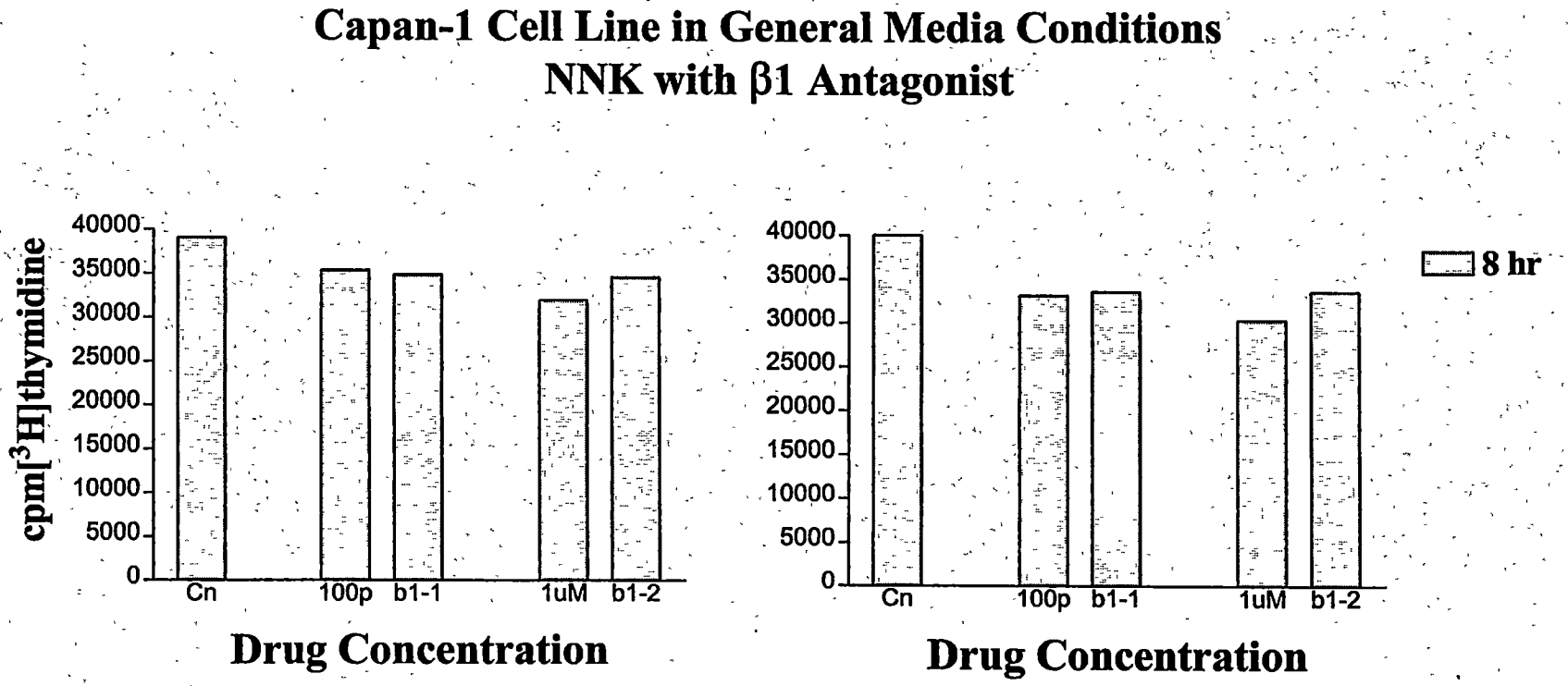

Figure 14: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B1-1=10 nM atenolol + 100 pM NNK. B1-2=100 μM atenolol + 1 μM NNK. The only statistically significant finding was the higher concentration of NNK and β1 antagonist resulted in a decrease compared to control, <0.05, in both run 1 and 2.

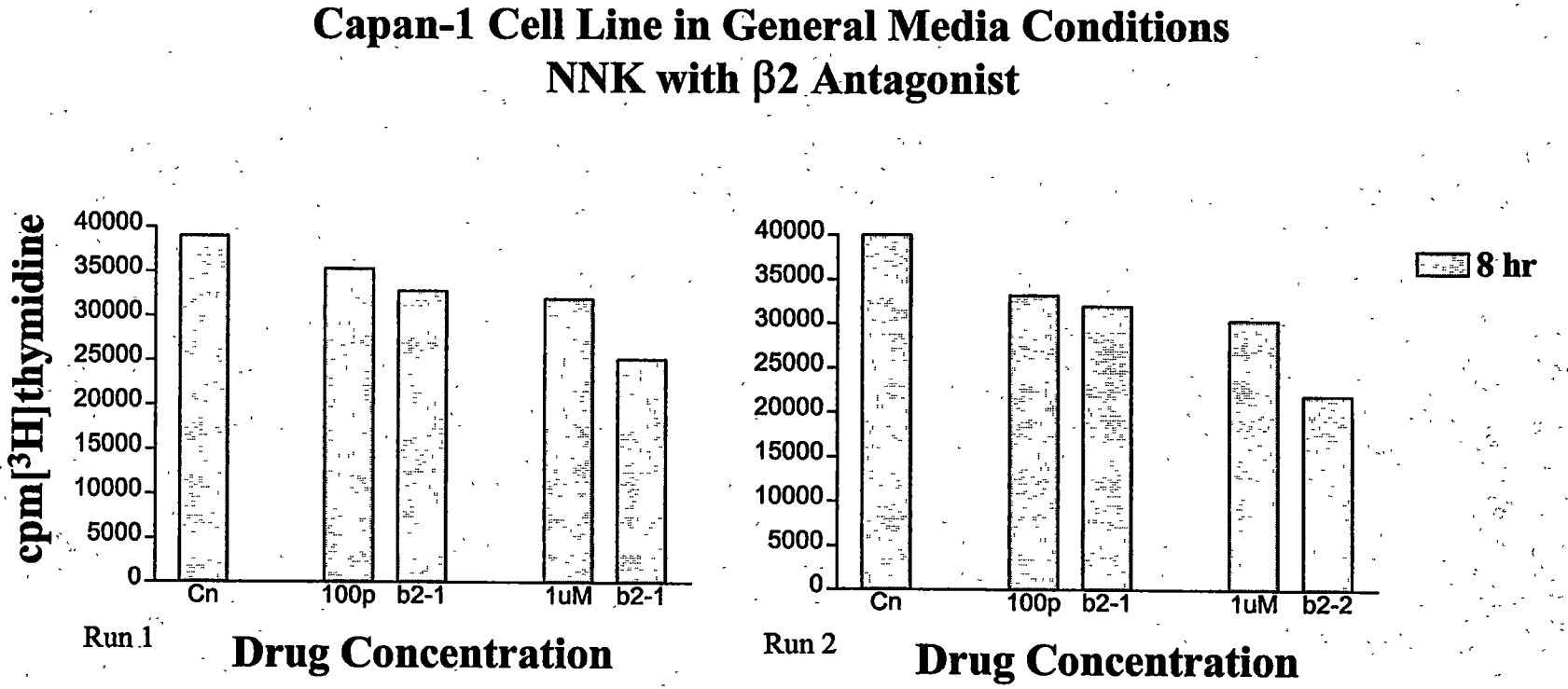

Figure 15: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B2-1=10 nM ICI 118,551 + 100 pM NNK. B2-2=100 μM ICI 118,551 + 1 μM NNK. In both run 1 and run 2, the higher concentration of NNK and the β2 antagonist resulted in a statistically significant decrease compared to NNK alone, $p<0.001$. The lower and higher concentrations resulted in statistically significant decreases compared to control, $p<0.01$.

Capan-1 Cell Line in Ethanol Media Conditions
Increasing Concentrations of NNK

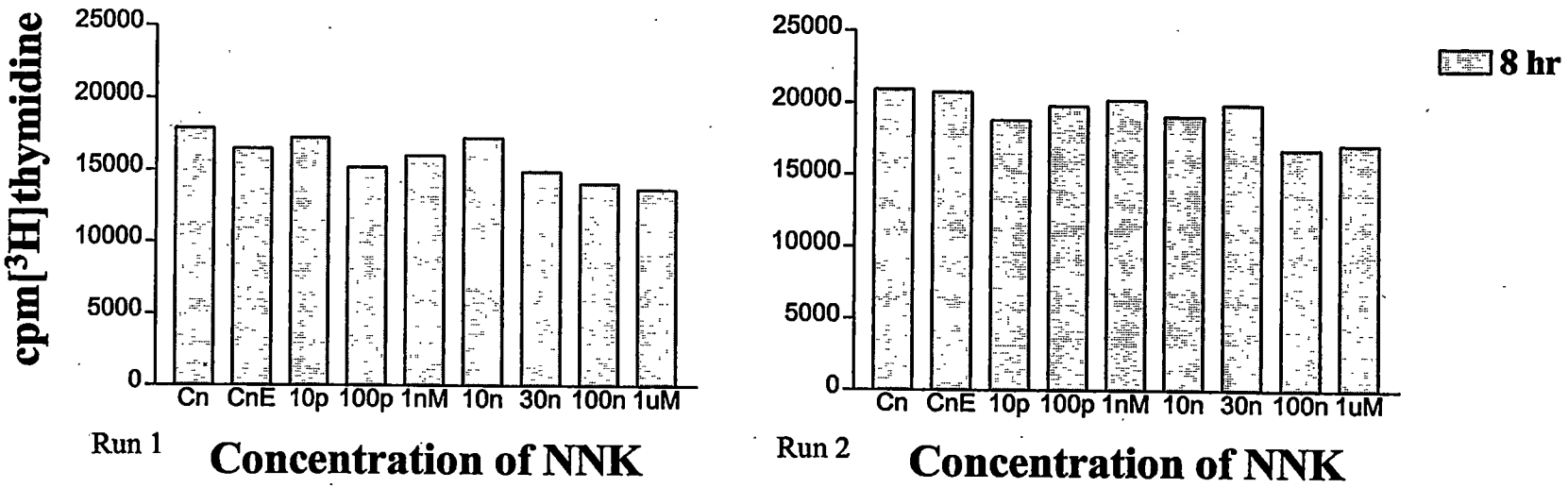

Figure 16: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of increasing concentrations of NNK in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown are represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in presence of PBS and no NNK. In run 1, the 2 highest concentrations of NNK represented aly significant from the ethanol control, $p<0.05$. In run 2, the 2 highest concentrations of NNK represented aly significant from the control, $p<0.01$, and the ethanol control, $p<0.01$.

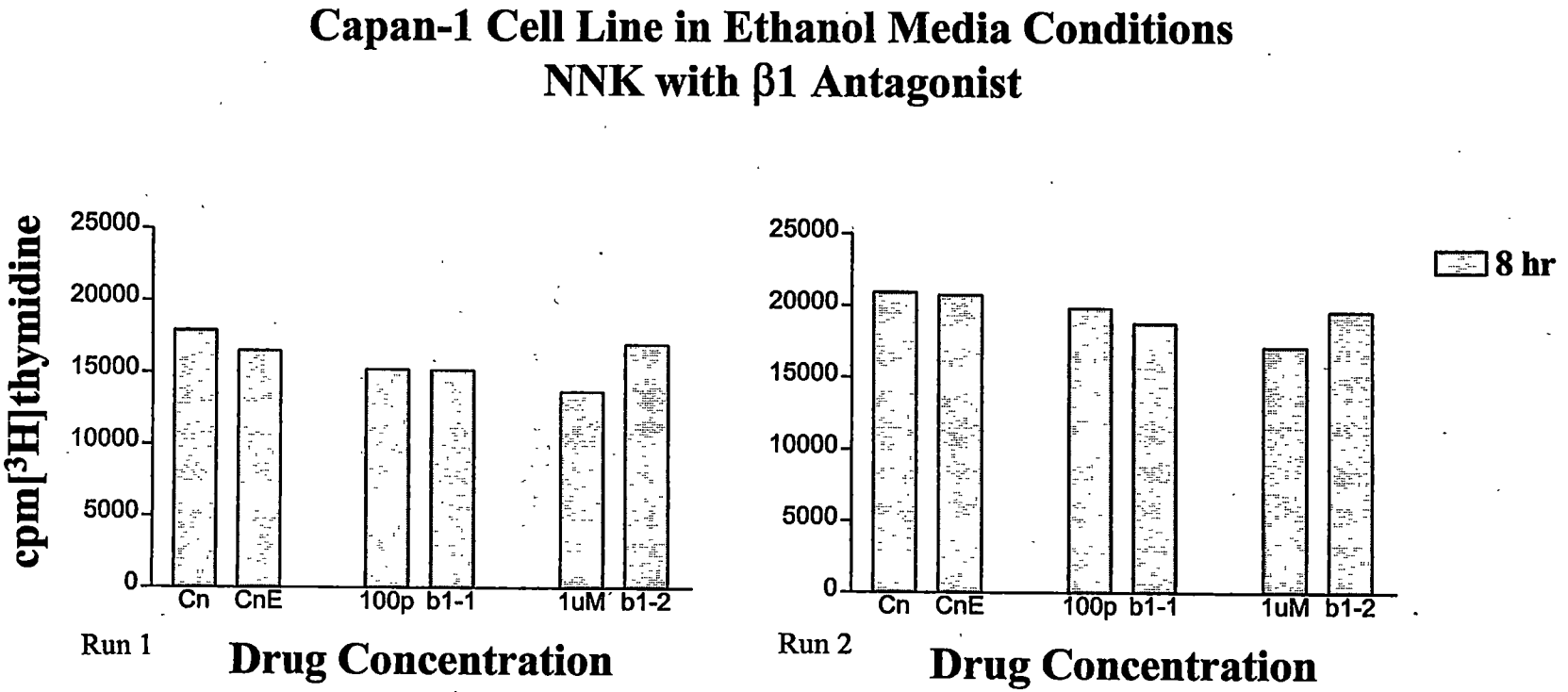

Figure 17: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β1 antagonist in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in the presence of PBS and no NNK. B1-1=10 nM atenolol + 100 pM NNK. B1-2=100 μM atenolol + 1 μM NNK. The only statistical finding was in run 2, the higher NNK and β1 antagonist concentration resulted a in statistically significant increase compared to NNK alone, $p<0.05$.

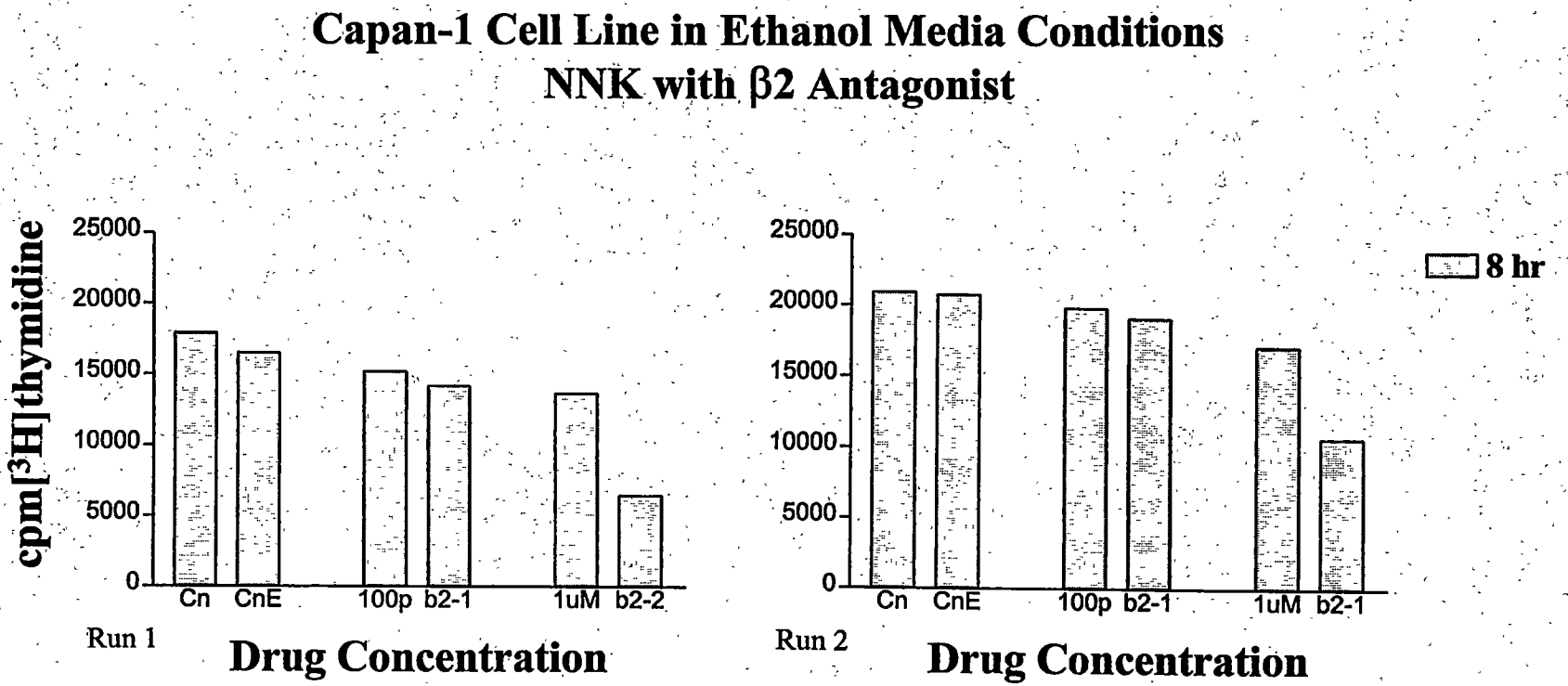

Figure 18: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B2-1=10 nM ICI 118,551 + 100 pM NNK. B2-2=100μM ICI 118,551 + 1 μM NNK. In both run 1 and run 2, the higher concentrations of NNK and the β2 antagonist resulted in a statistically significant decrease compared to NNK alone, $p<0.001$, and when compared to control and ethanol control, $p<0.01$.

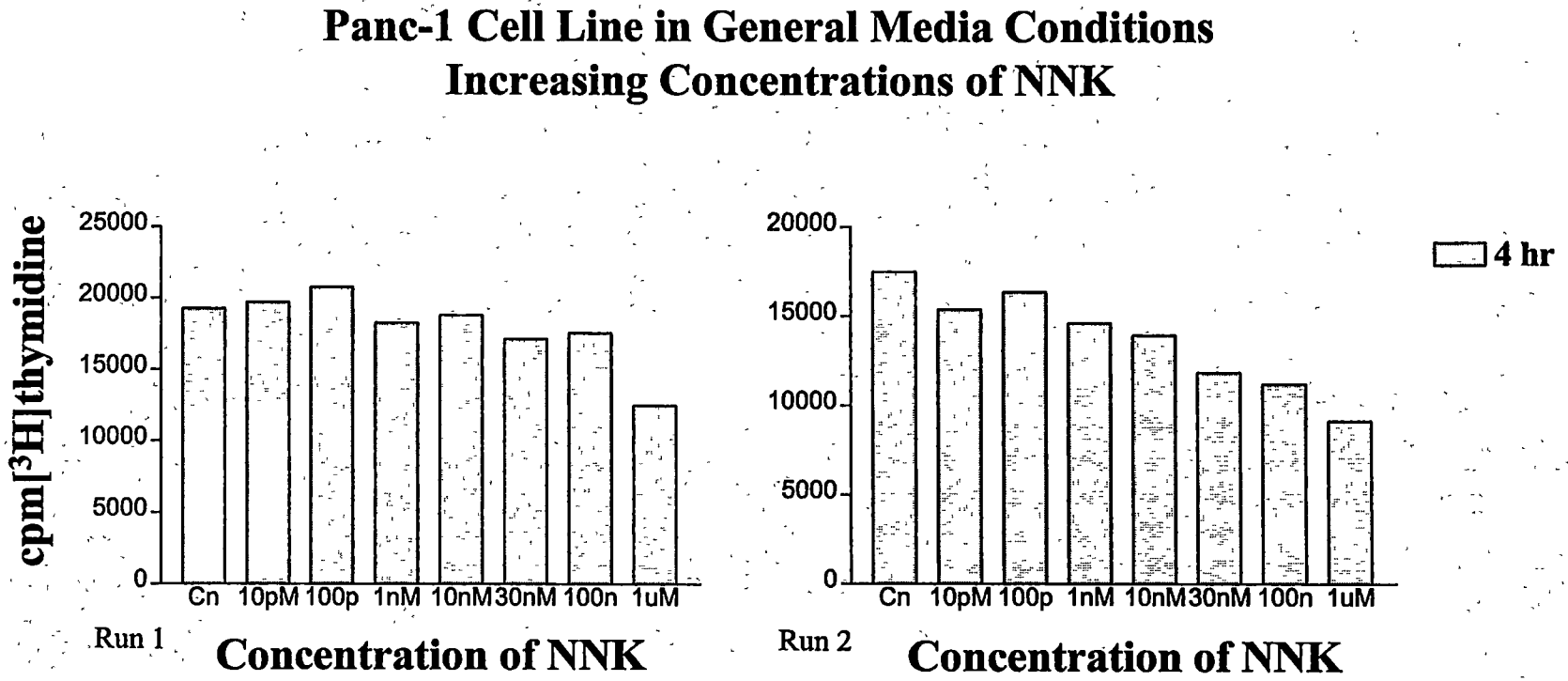

Figure 19: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of increasing concentrations of NNK in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. In run 1, the highest concentration of NNK resulted in a statistically significant decrease, $p<0.01$, compared to the control. In run 2, the 5 higher concentrations of NNK resulted in statistically significant decreases compared to control (1 nM=p,0.05; remaining higher concentrations=$p<0.01$).

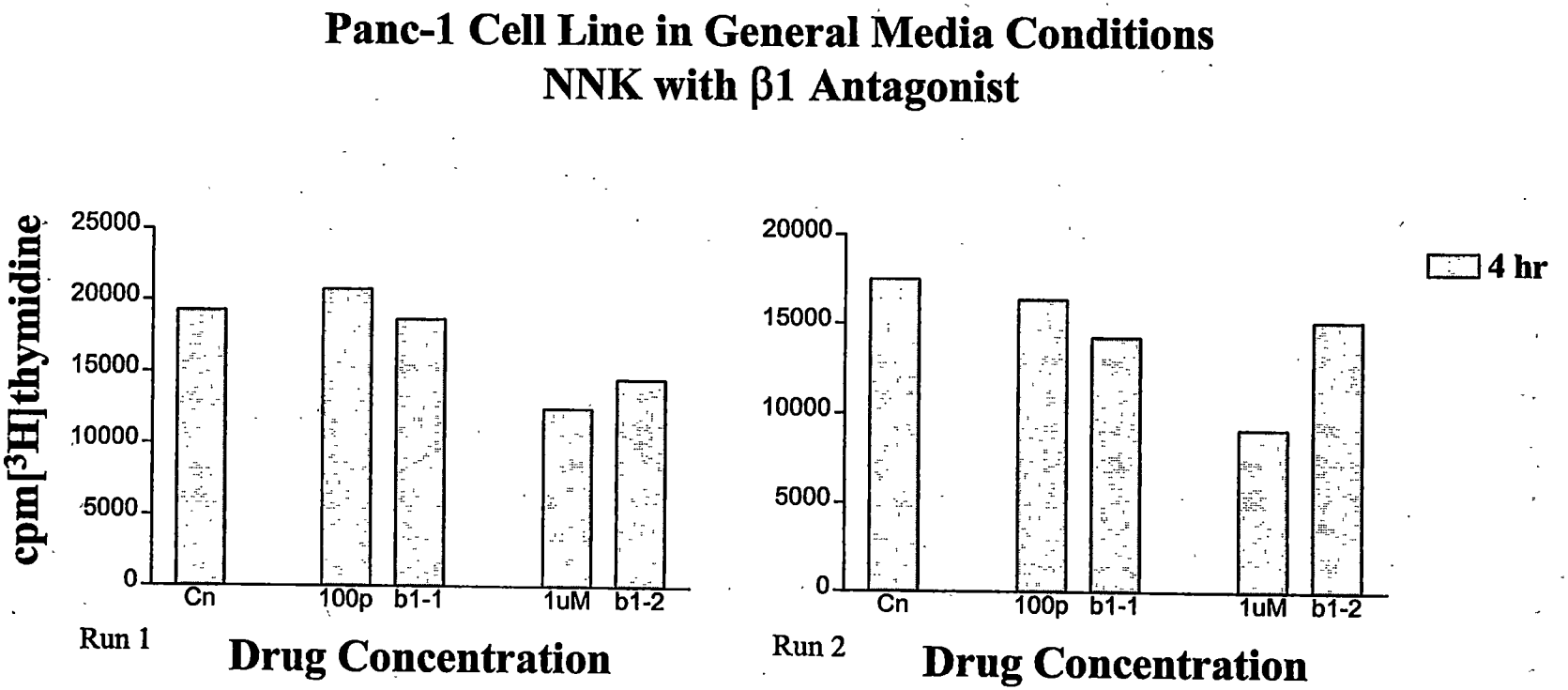

Figure 20: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β1 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B1-1=10 nM atenolol + 100 pM NNK. B1-2=100 μM atenolol + 1μM NNK. In run 1, the highest concentration of NNK resulted in a statistically significant decrease, $p<0.01$, compared to the control. In only one run (2), the lower and higher concentrations of NNK and the β1 antagonist resulted in statistically significant decreases, $p<0.05$ and $p<0.001$ respectively, compared to NNK alone.

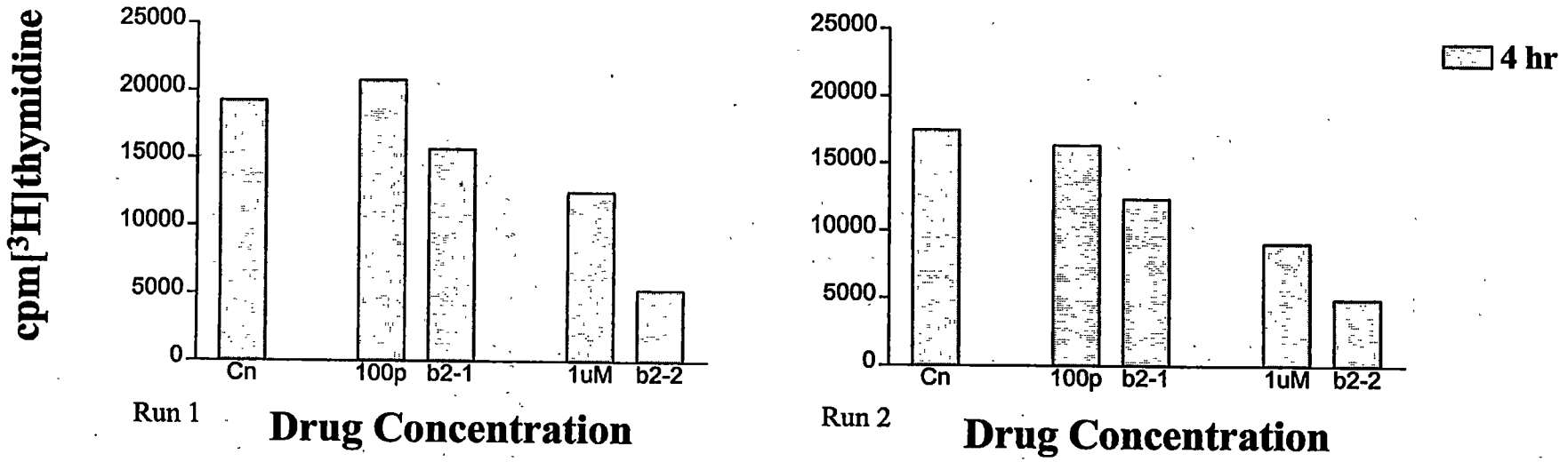

Figure 21: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in general media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. B2-1=10 nM ICI 118,551 + 100 pM NNK. B2-2=100μM ICI 118,551 + 1 μM NNK. In both run 1 and run 2, the lower and higher concentrations of NNK and the β2 antagonist resulted in a statistically significant decrease compared to NNK alone, $p<0.001$, and when compared to control, $p<0.05$ and $p<0.01$.

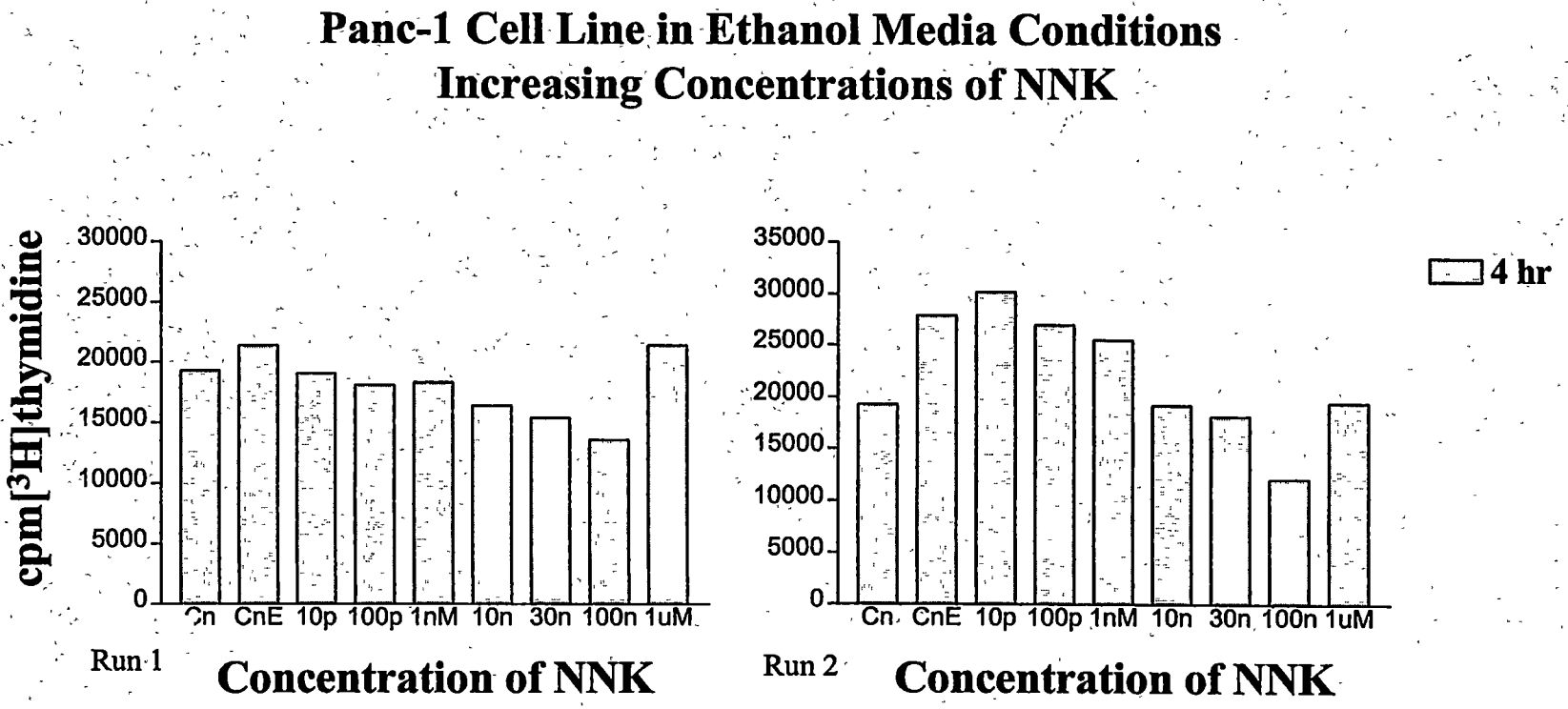

Figure 22: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of increasing concentrations of NNK in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown are represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in presence of PBS and no NNK. In run 1, there were no statistically significant findings. In run 2, there was a statistically significant increase in the ethanol control group compared to the control, $p<0.01$, and statistically significant increases in the 3 lower NNK concentrations compared to the control, $p<0.01$. Also in run 2, the 100 nM concentration was statistically decreased compared to the control, $p<0.01$. In run 2 only, the 4 highest concentrations were statistically decreased compared to the ethanol control, $p<0.01$.

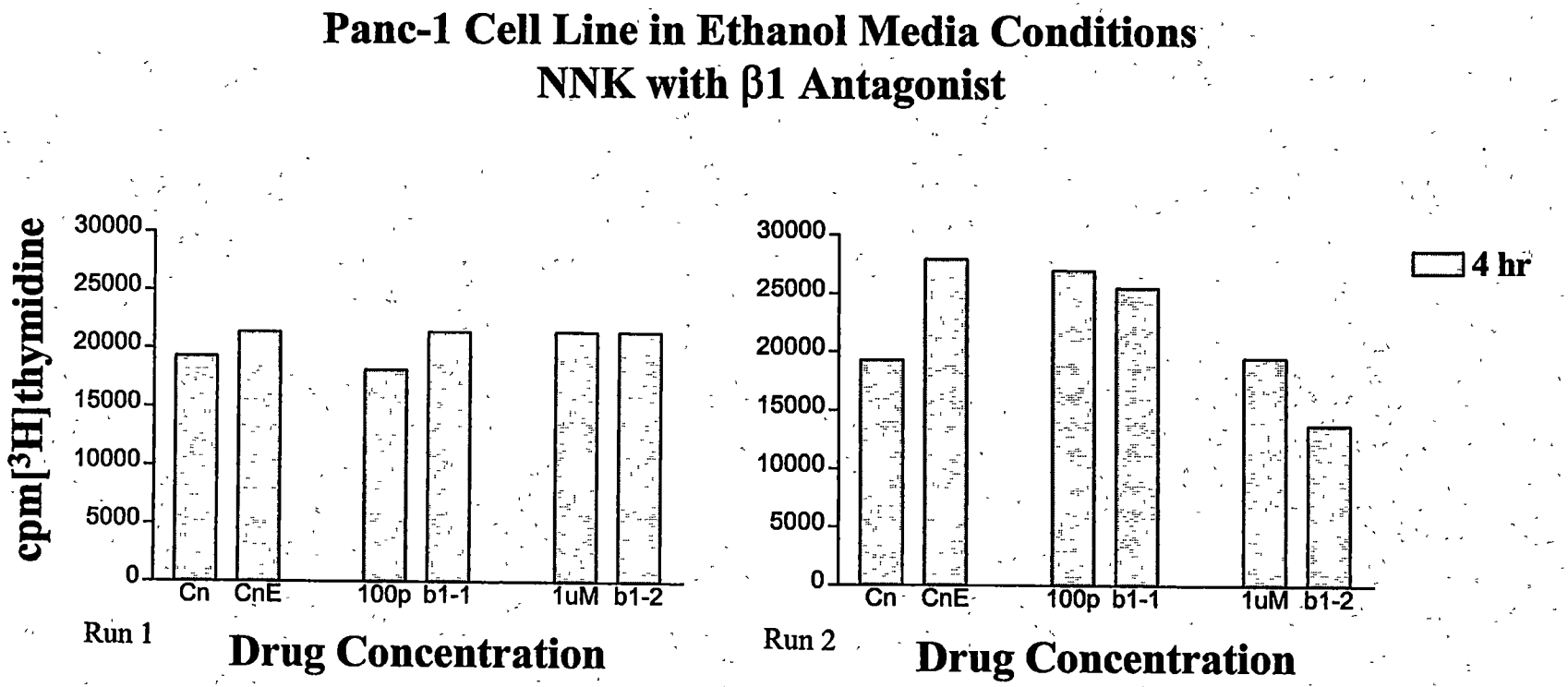

Figure 23: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β1 antagonist in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in the presence of PBS and no NNK. B1-1=10 nM atenolol + 100 pM NNK. B1-2=100 μM atenolol + 1 μM NNK. In only one run (2), both NNK and β1 antagonist concentrations resulted in statistically significant decreases compared to NNK alone or to the control and ethanol control groups, $p<0.001$.

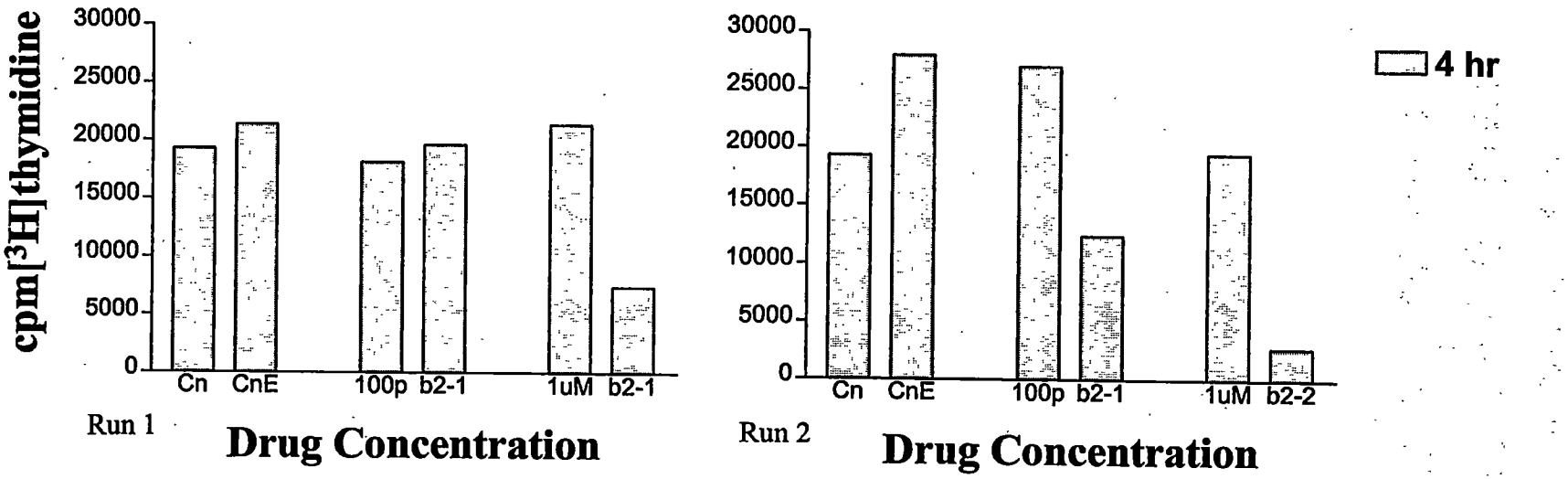

Figure 24: Results of DNA synthesis as a function of tritiated thymidine incorporation in the presence of NNK and a β2 antagonist in ethanol media conditions. Bars represent mean of each treatment group. Graphs shown represent two consecutive runs. Cn=control group in the presence of PBS and no NNK. CnE=control in ethanol conditions in presence of PBS and no NNK. B2-1=10 nM ICI 118,551 + 100 pM NNK. B2-2=100 μM ICI 118,551 + 1 μM NNK. In both run 1 and run 2, the higher concentration of NNK and the β2 antagonist resulted in a statistically significant decrease compared to NNK alone, $p<0.001$, and compared to control and ethanol control, $p<0.01$. In run 2, these findings were also true for the lower concentration of NNK and the β2 antagonist.

APPENDIX B

STATISTICAL DATA

BxPC-3 CELL LINE

GENERAL MEDIA CONDITIONS

BxPC-3 Cell Line in General Media Conditions
ANOVA with Dunnett Post Test; 8 hr Data
Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Contl vs 10p	-4757	1.213	P > 0.05	-15860 to 6343
Contl vs 100p	6118	1.560	P > 0.05	-4982 to 17220
Contl vs 1n	-2526	0.6440	P > 0.05	-13630 to 8574
Contl vs 10n	-238.2	0.06072	P > 0.05	-11340 to 10860
Contl vs 30n	-8110	2.068	P > 0.05	-19210 to 2990
Contl vs 100n	-6369	1.624	P > 0.05	-17470 to 4730
Contl vs 1u	-7190	1.833	P > 0.05	-18290 to 3910
Contl vs b1-1	-702.8	0.1792	P > 0.05	-11800 to 10400
Contl vs b1-2	-4692	1.196	P > 0.05	-15790 to 6408
Contl vs b2-1	2287	0.5830	P > 0.05	-8813 to 13390
Contl vs b2-2	30270	7.719	P < 0.01	19170 to 41370

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	49.63	0.01959	P > 0.05	-7136 to 7236
Cntl vs 100p	697.3	0.2886	P > 0.05	-6154 to 7549
Cntl vs 1n	-1824	0.7549	P > 0.05	-8675 to 5028
Cntl vs 10n	-940.3	0.3892	P > 0.05	-7792 to 5911
Cntl vs 30n	-2477	1.025	P > 0.05	-9328 to 4375
Cntl vs 100n	1899	0.7862	P > 0.05	-4952 to 8751
Cntl vs 1u	-6360	2.510	P > 0.05	-13550 to 825.6
Cntl vs b1-1	-1154	0.4776	P > 0.05	-8005 to 5698
Cntl vs b1-2	-162.5	0.06726	P > 0.05	-7014 to 6689
Cntl vs b2-1	-3808	1.503	P > 0.05	-10990 to 3378
Cntl vs b2-2	26820	11.10	P < 0.01	19970 to 33670

Run 2

Legend:

Cntl=control with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1uM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1uM NNK

BxPC-3 Cell Line in General Media Conditions ANOVA with Dunnett Post Test; 24 hr Data Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	-28630	3.237	P < 0.05	-53700 to -3561
Cntl vs 100p	-12930	1.462	P > 0.05	-38000 to 12140
Cntl vs 1n	-30780	3.331	P < 0.05	-56960 to -4592
Cntl vs 10n	-38700	4.374	P < 0.01	-63770 to -13620
Cntl vs 30n	-37370	4.224	P < 0.01	-62440 to -12300
Cntl vs 100n	-28190	3.186	P < 0.05	-53260 to -3117
Cntl vs 1u	-38080	4.305	P < 0.01	-63150 to -13010
Cntl vs b1-1	-17010	1.923	P > 0.05	-42080 to 8058
Cntl vs b1-2	-15760	1.782	P > 0.05	-40840 to 9307
Cntl vs b2-1	-18160	2.053	P > 0.05	-43230 to 6913
Cntl vs b2-2	80000	9.044	P < 0.01	54930 to 105100

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	6970	1.057	P > 0.05	-11700 to 25640
Cntl vs 100p	5414	0.8207	P > 0.05	-13250 to 24080
Cntl vs 1n	2175	0.3297	P > 0.05	-16490 to 20840
Cntl vs 10n	3389	0.5137	P > 0.05	-15280 to 22060
Cntl vs 30n	-2122	0.3217	P > 0.05	-20790 to 16550
Cntl vs 100n	-668.5	0.1013	P > 0.05	-19340 to 18000
Cntl vs 1u	10030	1.521	P > 0.05	-8634 to 28700
Cntl vs b1-1	17380	2.635	P > 0.05	-1286 to 36050
Cntl vs b1-2	19660	2.980	P < 0.05	988.9 to 38320
Cntl vs b2-1	257.5	0.03904	P > 0.05	-18410 to 18930
Cntl vs b2-2	112100	17.00	P < 0.01	93460 to 130800

Run 2

Legend:

Cntl=control with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1uM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1uM NNK

BxPC-3 Cell Line in General Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b1-1	-6821	1.739	P > 0.05	-15840 to 2197
1u vs b1-2	2498	0.6370	P > 0.05	-6519 to 11520

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b1-1	-1851	0.7662	P > 0.05	-7413 to 3711
1u vs b1-2	6198	2.446	P < 0.05	364.6 to 12030

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

BxPC-3 Cell Line in General Media Conditions
ANOVA with Bonferroni Multiple Comparison Test; 24 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b1-1	11970	1.814	P > 0.05	-3197 to 27130
1u vs b1-2	9623	1.459	P > 0.05	-5542 to 24790

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b1-1	-4082	0.4839	P > 0.05	-23490 to 15330
1u vs b1-2	22320	2.646	P < 0.05	2908 to 41730

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

BxPC-3 Cell Line in General Media Conditions ANOVA with Bonferroni Multiple Comparison Test; 8 hr Data NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b2-1	-3831	0.9768	P > 0.05	-12850 to 5186
1u vs b2-2	37460	9.552	P < 0.001	28450 to 46480

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b2-1	-4505	1.778	P > 0.05	-10340 to 1328
1u vs b2-2	33180	13.09	P < 0.001	27340 to 39010

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

BxPC-3 Cell Line in General Media Conditions ANOVA with Bonferroni Multiple Comparison Test; 24 hr Data NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b2-1	-5227	0.6197	P > 0.05	-24640 to 14180
1u vs b2-2	118100	14.00	P < 0.001	98680 to 137500

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b2-1	-5156	0.7817	P > 0.05	-20320 to 10010
1u vs b2-2	102100	15.48	P < 0.001	86930 to 117300

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

BxPC-3 CELL LINE

ETHANOL MEDIA CONDITIONS

BxPC-3 Cell Line in Ethanol Media Conditions
ANOVA with Dunnett Post Test; 8 hr Data
Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	-804.5	0.3040	P > 0.05	-8389 to 6780
Cn vs 10p	798.2	0.3016	P > 0.05	-6787 to 8383
Cn vs 100p	424.2	0.1528	P > 0.05	-7531 to 8379
Cn vs 1n	3992	1.349	P > 0.05	-4488 to 12470
Cn vs 10n	3416	1.291	P > 0.05	-4169 to 11000
Cn vs 30n	2505	0.8467	P > 0.05	-5975 to 10990
Cn vs 100n	2818	1.065	P > 0.05	-4767 to 10400
Cn vs 1u	2922	1.104	P > 0.05	-4663 to 10510
Cn vs B1-1	3234	1.222	P > 0.05	-4351 to 10820
Cn vs B1-2	8532	3.074	P < 0.05	577.3 to 16490
Cn vs B2-1	7923	2.678	P > 0.05	-556.9 to 16400
Cn vs B2-2	20270	7.661	P < 0.01	12690 to 27860

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	2374	0.8205	P > 0.05	-5897 to 10650
Cn vs 10p	3879	1.341	P > 0.05	-4392 to 12150
Cn vs 100p	5322	1.839	P > 0.05	-2949 to 13590
Cn vs 1n	2455	0.8483	P > 0.05	-5816 to 10730
Cn vs 10n	6967	2.408	P > 0.05	-1304 to 15240
Cn vs 30n	16900	5.839	P < 0.01	8624 to 25170
Cn vs 100n	4732	1.635	P > 0.05	-3539 to 13000
Cn vs 1u	6571	2.165	P > 0.05	-2104 to 15250
Cn vs B1-1	5079	1.673	P > 0.05	-3596 to 13750
Cn vs B1-2	9266	3.202	P < 0.05	994.9 to 17540
Cn vs B2-1	6467	2.131	P > 0.05	-2208 to 15140
Cn vs B2-2	25790	8.914	P < 0.01	17520 to 34060

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

BxPC-3 Cell Line in Ethanol Media Conditions
ANOVA with Dunnett Post Test; 24 hr Data
Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	-2706	0.4798	P > 0.05	-18950 to 13540
Cn vs 10p	4598	0.8153	P > 0.05	-11640 to 20840
Cn vs 100p	9572	1.773	P > 0.05	-5978 to 25120
Cn vs 1n	25480	4.719	P < 0.01	9931 to 41030
Cn vs 10n	27790	4.647	P < 0.01	10570 to 45020
Cn vs 30n	25810	4.316	P < 0.01	8588 to 43040
Cn vs 100n	31680	4.865	P < 0.01	12930 to 50430
Cn vs 1u	34960	5.844	P < 0.01	17730 to 52180
Cn vs B1-1	15090	2.795	P > 0.05	-460.6 to 30640
Cn vs B1-2	13410	2.378	P > 0.05	-2830 to 29650
Cn vs B2-1	18360	3.401	P < 0.05	2814 to 33910
Cn vs B2-2	68840	11.51	P < 0.01	51620 to 86070

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	59760	10.85	P < 0.01	43930 to 75600
Cn vs 10p	62360	11.32	P < 0.01	46520 to 78190
Cn vs 100p	65880	10.24	P < 0.01	47390 to 84380
Cn vs 1n	66360	12.63	P < 0.01	51260 to 81460
Cn vs 10n	72740	13.20	P < 0.01	56900 to 88570
Cn vs 30n	87760	15.93	P < 0.01	71930 to 103600
Cn vs 100n	97290	17.66	P < 0.01	81460 to 113100
Cn vs 1u	87990	15.97	P < 0.01	72160 to 103800
Cn vs B1-1	66250	12.61	P < 0.01	51150 to 81340
Cn vs B1-2	72560	12.35	P < 0.01	55680 to 89440
Cn vs B2-1	69840	13.29	P < 0.01	54740 to 84940
Cn vs B2-2	106500	19.33	P < 0.01	90680 to 122300

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

BxPC-3 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 8 hr Data Ethanol Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10p	1603	0.5953	P > 0.05	-6060 to 9265
CnE vs 100p	1229	0.4351	P > 0.05	-6808 to 9265
CnE vs 1n	4797	1.594	P > 0.05	-3770 to 13360
CnE vs 10n	4221	1.568	P > 0.05	-3442 to 11880
CnE vs 30n	3310	1.100	P > 0.05	-5257 to 11880
CnE vs 100n	3622	1.345	P > 0.05	-4040 to 11280
CnE vs 1u	3726	1.384	P > 0.05	-3936 to 11390
CnE vs B1-1	4038	1.500	P > 0.05	-3624 to 11700
CnE vs B1-2	9337	3.307	P < 0.05	1300 to 17370
CnE vs B2-1	8728	2.899	P < 0.05	160.7 to 17290
CnE vs B2-2	21080	7.829	P < 0.01	13420 to 28740

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10p	1505	0.5188	P > 0.05	-6723 to 9733
CnE vs 100p	2948	1.016	P > 0.05	-5280 to 11180
CnE vs 1n	80.50	0.02775	P > 0.05	-8147 to 8308
CnE vs 10n	4592	1.583	P > 0.05	-3636 to 12820
CnE vs 30n	14520	5.005	P < 0.01	6293 to 22750
CnE vs 100n	2358	0.8126	P > 0.05	-5870 to 10590
CnE vs 1u	4197	1.379	P > 0.05	-4433 to 12830
CnE vs B1-1	2704	0.8888	P > 0.05	-5925 to 11330
CnE vs B1-2	6892	2.375	P > 0.05	-1336 to 15120
CnE vs B2-1	4092	1.345	P > 0.05	-4537 to 12720
CnE vs B2-2	23420	8.072	P < 0.01	15190 to 31650

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

BxPC-3 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 24 hr Data Ethanol Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10p	7304	1.276	P > 0.05	-9053 to 23660
CnE vs 100p	12280	2.241	P > 0.05	-3382 to 27940
CnE vs 1n	28190	5.144	P < 0.01	12530 to 43850
CnE vs 10n	30500	5.024	P < 0.01	13150 to 47850
CnE vs 30n	28520	4.698	P < 0.01	11170 to 45870
CnE vs 100n	34380	5.203	P < 0.01	15500 to 53270
CnE vs 1u	37660	6.204	P < 0.01	20310 to 55010
CnE vs B1-1	17790	3.248	P < 0.05	2135 to 33450
CnE vs B1-2	16120	2.816	P > 0.05	-239.9 to 32470
CnE vs B2-1	21070	3.845	P < 0.01	5409 to 36730
CnE vs B2-2	71550	11.79	P < 0.01	54200 to 88900

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10p	2596	0.4925	P > 0.05	-12450 to 17640
CnE vs 100p	6122	1.006	P > 0.05	-11250 to 23500
CnE vs 1n	6597	1.307	P > 0.05	-7809 to 21000
CnE vs 10n	12980	2.461	P > 0.05	-2072 to 28020
CnE vs 30n	28000	5.311	P < 0.01	12950 to 43050
CnE vs 100n	37530	7.119	P < 0.01	22490 to 52580
CnE vs 1u	28230	5.355	P < 0.01	13180 to 43280
CnE vs B1-1	6485	1.285	P > 0.05	-7922 to 20890
CnE vs B1-2	12800	2.289	P > 0.05	-3160 to 28760
CnE vs B2-1	10080	1.997	P > 0.05	-4326 to 24490
CnE vs B2-2	46750	8.867	P < 0.01	31700 to 61800

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

BxPC-3 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	2809	1.012	P > 0.05	-3580 to 9199
1u vs B1-2	5611	2.021	P > 0.05	-779.2 to 12000

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	-243.0	0.08008	P > 0.05	-7215 to 6728
1u vs B1-2	2695	0.8880	P > 0.05	-4276 to 9666

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

BxPC-3 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
24 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	5517	1.072	P > 0.05	-6379 to 17410
1u vs B1-2	-21550	3.602	P < 0.01	-35370 to -7723

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	362.7	0.05636	P > 0.05	-14480 to 15210
1u vs B1-2	-15430	2.528	P < 0.05	-29510 to -1350

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

BxPC-3 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	7499	2.439	P < 0.05	420.0 to 14580
1u vs B2-2	17350	6.557	P < 0.001	11260 to 23440

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	1145	0.3773	P > 0.05	-5827 to 8116
1μ vs B2-2	19220	6.334	P < 0.001	12250 to 26190

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

BxPC-3 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
24 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	8791	1.708	P > 0.05	-3105 to 20690
1u vs B2-2	33890	5.375	P < 0.001	19320 to 48460

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	3959	0.6153	P > 0.05	-10880 to 18800
1u vs B2-2	18520	3.218	P < 0.01	5243 to 31800

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

AsPC-1 CELL LINE

GENERAL MEDIA CONDITIONS

AsPC-1 Cell Line in General Media Conditions ANOVA with Dunnett Post Test; 8 hr Data Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	-4454	1.418	P > 0.05	-13400 to 4489
Cntl vs 100p	-4571	1.261	P > 0.05	-14900 to 5754
Cntl vs 1n	4097	1.230	P > 0.05	-5388 to 13580
Cntl vs 10n	814.7	0.2710	P > 0.05	-7747 to 9376
Cntl vs 30n	265.8	0.08465	P > 0.05	-8677 to 9208
Cntl vs 100n	6387	2.125	P > 0.05	-2174 to 14950
Cntl vs 1u	2407	0.8006	P > 0.05	-6155 to 10970
Cntl vs B1-1	483.0	0.1607	P > 0.05	-8079 to 9045
Cntl vs B1-2	6555	2.088	P > 0.05	-2387 to 15500
Cntl vs B2-1	3660	1.217	P > 0.05	-4902 to 12220
Cntl vs B2-2	32100	10.68	P < 0.01	23540 to 40660

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	1954	0.7726	P > 0.05	-5240 to 9148
Cntl vs 100p	4506	1.781	P > 0.05	-2688 to 11700
Cntl vs 1n	851.8	0.3040	P > 0.05	-7117 to 8821
Cntl vs 10n	2518	0.9956	P > 0.05	-4675 to 9712
Cntl vs 30n	1188	0.4496	P > 0.05	-6326 to 8701
Cntl vs 100n	5607	2.217	P > 0.05	-1587 to 12800
Cntl vs 1u	5273	1.996	P > 0.05	-2241 to 12790
Cntl vs B1-1	5689	2.153	P > 0.05	-1825 to 13200
Cntl vs B1-2	5998	2.371	P > 0.05	-1195 to 13190
Cntl vs B2-1	7495	2.837	P > 0.05	-18.11 to 15010
Cntl vs B2-2	29560	11.69	P < 0.01	22360 to 36750

Run 2

Legend:

Cntl=control with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1uM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1uM NNK

AsPC-1 Cell Line in General Media Conditions ANOVA with Dunnett Post Test; 24 hr Data Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	7030	1.354	P > 0.05	-7703 to 21760
Cntl vs 100p	9992	1.925	P > 0.05	-4741 to 24720
Cntl vs 1n	14120	2.721	P > 0.05	-609.4 to 28860
Cntl vs 10n	2958	0.5096	P > 0.05	-13510 to 19430
Cntl vs 30n	12240	2.249	P > 0.05	-3209 to 27690
Cntl vs 100n	9816	1.891	P > 0.05	-4917 to 24550
Cntl vs 1u	9841	1.896	P > 0.05	-4892 to 24570
Cntl vs B1-1	8139	1.568	P > 0.05	-6594 to 22870
Cntl vs B1-2	8835	1.623	P > 0.05	-6617 to 24290
Cntl vs B2-1	6098	1.175	P > 0.05	-8635 to 20830
Cntl vs B2-2	42050	8.101	P < 0.01	27320 to 56790

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	9129	1.511	P > 0.05	-8059 to 26320
Cntl vs 100p	801.9	0.1327	P > 0.05	-16390 to 17990
Cntl vs 1n	18050	2.987	P < 0.05	863.1 to 35240
Cntl vs 10n	12560	2.078	P > 0.05	-4629 to 29750
Cntl vs 30n	5725	0.9472	P > 0.05	-11460 to 22910
Cntl vs 100n	2680	0.4003	P > 0.05	-16360 to 21720
Cntl vs 1u	12700	2.013	P > 0.05	-5248 to 30660
Cntl vs B1-1	12440	2.058	P > 0.05	-4748 to 29630
Cntl vs B1-2	9590	1.519	P > 0.05	-8363 to 27540
Cntl vs B2-1	4509	0.7142	P > 0.05	-13440 to 22460
Cntl vs B2-2	53980	8.551	P < 0.01	36030 to 71930

Run 2

Legend:

Cntl=control with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1uM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1uM NNK

AsPC-1 Cell Line in General Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	5054	1.440	P > 0.05	-3053 to 13160
1u vs B1-2	4149	1.380	P > 0.05	-2794 to 11090

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	1183	0.4676	P > 0.05	-4652 to 7018
1u vs B1-2	725.5	0.2868	P > 0.05	-5110 to 6560

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

AsPC-1 Cell Line in General Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
24 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	-1853	0.3569	P > 0.05	-13810 to 10100
1u vs B1-2	-1005	0.1846	P > 0.05	-13550 to 11540

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	11640	2.020	P > 0.05	-1655 to 24930
1u vs B1-2	-3114	0.4933	P > 0.05	-17680 to 11450

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

AsPC-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	8231	2.345	P < 0.05	123.8 to 16340
1u vs B2-2	29690	10.36	P < 0.001	23070 to 36310

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	2989	1.182	P > 0.05	-2846 to 8824
1u vs B2-2	24280	9.600	P < 0.001	18450 to 30120

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

AsPC-1 Cell Line in General Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
24 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	-3894	0.7501	P > 0.05	-15850 to 8063
1u vs B2-2	32210	6.205	P < 0.001	20260 to 44170

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	3707	0.6133	P > 0.05	-10240 to 17650
1u vs B2-2	41280	6.539	P < 0.001	26710 to 55840

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

AsPC-1 CELL LINE

ETHANOL MEDIA CONDITIONS

AsPC-1 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 8 hr Data Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	-2093	0.6822	P > 0.05	-10970 to 6780
Cn vs 10pM	-12050	4.473	P < 0.01	-19850 to -4261
Cn vs 100p	-5840	2.056	P > 0.05	-14050 to 2375
Cn vs 1nM	-8559	3.013	P < 0.05	-16770 to -343.4
Cn vs 10nM	-7040	2.612	P > 0.05	-14830 to 753.3
Cn vs 30n	-4820	1.697	P > 0.05	-13030 to 3396
Cn vs 100n	-2293	0.8070	P > 0.05	-10510 to 5923
Cn vs 1uM	-6886	2.244	P > 0.05	-15760 to 1987
Cn vs B1-2	-2719	1.049	P > 0.05	-10220 to 4780
Cn vs B1-2	2493	0.9614	P > 0.05	-5006 to 9993
Cn vs B2-1	-2015	0.7476	P > 0.05	-9808 to 5779
Cn vs B2-2	25930	9.128	P < 0.01	17710 to 34140

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	-2093	0.6822	P > 0.05	-10970 to 6780
Cn vs 10pM	-12050	4.473	P < 0.01	-19850 to -4261
Cn vs 100p	-5840	2.056	P > 0.05	-14050 to 2375
Cn vs 1nM	-8559	3.013	P < 0.05	-16770 to -343.4
Cn vs 10nM	-7040	2.612	P > 0.05	-14830 to 753.3
Cn vs 30n	-4820	1.697	P > 0.05	-13030 to 3396
Cn vs 100n	-2293	0.8070	P > 0.05	-10510 to 5923
Cn vs 1uM	-6886	2.244	P > 0.05	-15760 to 1987
Cn vs B1-2	-2719	1.049	P > 0.05	-10220 to 4780
Cn vs B1-2	2493	0.9614	P > 0.05	-5006 to 9993
Cn vs B2-1	-2015	0.7476	P > 0.05	-9808 to 5779
Cn vs B2-2	25930	9.128	P < 0.01	17710 to 34140

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

AsPC-1 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 24 hr Data Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	43610	7.889	P < 0.01	27460 to 59750
Cn vs 10pM	30420	5.836	P < 0.01	15200 to 45630
Cn vs 100p	38100	6.331	P < 0.01	20530 to 55670
Cn vs 1nM	37180	7.451	P < 0.01	22610 to 51750
Cn vs 10nM	40980	6.810	P < 0.01	23410 to 58550
Cn vs 30n	37420	6.218	P < 0.01	19850 to 54990
Cn vs 100n	39600	7.599	P < 0.01	24380 to 54820
Cn vs 1uM	35550	5.157	P < 0.01	15420 to 55680
Cn vs B1-2	36960	6.143	P < 0.01	19390 to 54540
Cn vs B1-2	39580	5.741	P < 0.01	19450 to 59710
Cn vs B2-1	36080	5.996	P < 0.01	18510 to 53650
Cn vs B2-2	83590	16.04	P < 0.01	68370 to 98810

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	39850	10.13	P < 0.01	28530 to 51160
Cn vs 10pM	38640	10.98	P < 0.01	28520 to 48760
Cn vs 100p	39430	10.02	P < 0.01	28120 to 50750
Cn vs 1nM	42080	11.96	P < 0.01	31960 to 52200
Cn vs 10nM	52070	14.11	P < 0.01	41450 to 62680
Cn vs 30n	38120	10.33	P < 0.01	27510 to 48740
Cn vs 100n	43630	11.82	P < 0.01	33020 to 54250
Cn vs 1uM	42810	11.60	P < 0.01	32200 to 53430
Cn vs B1-2	42320	12.03	P < 0.01	32200 to 52450
Cn vs B1-2	50750	12.90	P < 0.01	39440 to 62070
Cn vs B2-1	46290	11.76	P < 0.01	34970 to 57600
Cn vs B2-2	93190	25.25	P < 0.01	82580 to 103800

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

AsPC-1 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 8 hr Data Ethanol Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10pM	-3442	1.662	P > 0.05	-9322 to 2438
CnE vs 100p	-4069	2.061	P > 0.05	-9676 to 1537
CnE vs 1nM	-1421	0.6861	P > 0.05	-7301 to 4460
CnE vs 10nM	-609.3	0.3087	P > 0.05	-6216 to 4997
CnE vs 30n	4044	1.953	P > 0.05	-1836 to 9924
CnE vs 100n	3350	1.697	P > 0.05	-2256 to 8957
CnE vs 1uM	8655	4.384	P < 0.01	3048 to 14260
CnE vs B1-2	914.8	0.4145	P > 0.05	-5353 to 7183
CnE vs B1-2	2794	1.415	P > 0.05	-2812 to 8401
CnE vs B2-1	3688	1.868	P > 0.05	-1918 to 9295
CnE vs B2-2	24060	12.19	P < 0.01	18450 to 29660

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10pM	-9961	3.309	P < 0.05	-18590 to -1328
CnE vs 100p	-3747	1.190	P > 0.05	-12780 to 5282
CnE vs 1nM	-6465	2.054	P > 0.05	-15490 to 2563
CnE vs 10nM	-4947	1.644	P > 0.05	-13580 to 3686
CnE vs 30n	-2726	0.8660	P > 0.05	-11750 to 6302
CnE vs 100n	-199.3	0.06332	P > 0.05	-9228 to 8829
CnE vs 1uM	-4793	1.424	P > 0.05	-14440 to 4859
CnE vs B1-2	-626.0	0.2148	P > 0.05	-8985 to 7733
CnE vs B1-2	4586	1.574	P > 0.05	-3772 to 12950
CnE vs B2-1	78.47	0.02607	P > 0.05	-8554 to 8711
CnE vs B2-2	28020	8.901	P < 0.01	18990 to 37050

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

AsPC-1 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 24 hr Data Ethanol Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10pM	-13190	2.354	P > 0.05	-29490 to 3115
CnE vs 100p	-5504	0.8628	P > 0.05	-24070 to 13060
CnE vs 1nM	-6430	1.193	P > 0.05	-22120 to 9259
CnE vs 10nM	-2626	0.4116	P > 0.05	-21190 to 15940
CnE vs 30n	-6186	0.9696	P > 0.05	-24750 to 12380
CnE vs 100n	-4004	0.7147	P > 0.05	-20310 to 12300
CnE vs 1uM	-8055	1.114	P > 0.05	-29100 to 12990
CnE vs B1-2	-6641	1.041	P > 0.05	-25200 to 11920
CnE vs B1-2	-4025	0.5564	P > 0.05	-25070 to 17020
CnE vs B2-1	-7524	1.179	P > 0.05	-26090 to 11040
CnE vs B2-2	39980	7.136	P < 0.01	23680 to 56290

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10pM	-1209	0.3001	P > 0.05	-12710 to 10290
CnE vs 100p	-417.3	0.09458	P > 0.05	-13020 to 12180
CnE vs 1nM	2233	0.5545	P > 0.05	-9269 to 13730
CnE vs 10nM	12220	2.919	P < 0.05	264.4 to 24170
CnE vs 30n	-1726	0.4124	P > 0.05	-13680 to 10230
CnE vs 100n	3784	0.9042	P > 0.05	-8169 to 15740
CnE vs 1uM	2964	0.7081	P > 0.05	-8989 to 14920
CnE vs B1-2	2477	0.6150	P > 0.05	-9025 to 13980
CnE vs B1-2	10910	2.472	P > 0.05	-1693 to 23510
CnE vs B2-1	6439	1.460	P > 0.05	-6161 to 19040
CnE vs B2-2	53340	12.75	P < 0.01	41390 to 65300

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

AsPC-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-2	4984	2.252	P > 0.05	-107.4 to 10080
1uM vs B1-2	-5861	2.960	P < 0.01	-10410 to -1307

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-2	3121	1.203	P > 0.05	-2897 to 9139
1uM vs B1-2	9379	3.302	P < 0.01	2787 to 15970

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

AsPC-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
24 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-2	-1136	0.1689	P > 0.05	-16870 to 14600
1uM vs B1-2	4031	0.4891	P > 0.05	-15240 to 23300

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-2	2894	0.7356	P > 0.05	-6187 to 11970
1uM vs B1-2	7943	1.943	P > 0.05	-1494 to 17380

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

AsPC-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
24 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	-2020	0.3002	P > 0.05	-17760 to 13720
1uM vs B2-2	48040	6.968	P < 0.001	31910 to 64170

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	6856	1.591	P > 0.05	-3091 to 16800
1uM vs B2-2	50380	13.07	P < 0.001	41480 to 59280

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

AsPC-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	7758	3.918	P < 0.001	3204 to 12310
1uM vs B2-2	15400	7.779	P < 0.001	10850 to 19960

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	3825	1.419	P > 0.05	-2429 to 10080
1uM vs B2-2	32820	10.70	P < 0.001	25690 to 39940

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

Capan-1 CELL LINE

GENERAL MEDIA CONDITIONS

Capan-1 Cell Line in General Media Conditions ANOVA with Dunnett Post Test; 8 hr Data Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	962.1	0.5915	P > 0.05	-3660 to 5584
Cntl vs 100p	3648	2.243	P > 0.05	-974.0 to 8271
Cntl vs 1n	2710	1.747	P > 0.05	-1697 to 7117
Cntl vs 10n	6230	3.830	P < 0.01	1607 to 10850
Cntl vs 30n	6266	4.041	P < 0.01	1859 to 10670
Cntl vs 100n	4571	2.947	P < 0.05	163.3 to 8978
Cntl vs 1u	7064	4.343	P < 0.01	2441 to 11690
Cntl vs B1-1	4181	2.696	P > 0.05	-226.7 to 8588
Cntl vs B1-2	4451	2.870	P < 0.05	43.68 to 8858
Cntl vs B2-1	6175	3.982	P < 0.01	1768 to 10580
Cntl vs B2-2	13840	7.983	P < 0.01	8914 to 18770

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	7016	3.661	P < 0.01	1592 to 12440
Cntl vs 100p	6885	3.592	P < 0.01	1461 to 12310
Cntl vs 1n	5217	2.722	P > 0.05	-206.9 to 10640
Cntl vs 10n	8022	4.185	P < 0.01	2598 to 13450
Cntl vs 30n	5113	2.668	P > 0.05	-311.2 to 10540
Cntl vs 100n	7771	4.055	P < 0.01	2347 to 13200
Cntl vs 1u	9717	5.070	P < 0.01	4293 to 15140
Cntl vs B1-1	6453	3.367	P < 0.05	1029 to 11880
Cntl vs B1-2	6456	3.368	P < 0.05	1032 to 11880
Cntl vs B2-1	8104	4.228	P < 0.01	2680 to 13530
Cntl vs B2-2	18220	9.505	P < 0.01	12790 to 23640

Run 2

Legend:

Cntl=control with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1uM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1uM NNK

Capan-1 Cell Line in General Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	532.2	0.3272	P > 0.05	-3218 to 4282
1u vs B1-2	-2613	1.606	P > 0.05	-6363 to 1137

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	-431.8	0.2253	P > 0.05	-4838 to 3975
1u vs B1-2	-3262	1.702	P > 0.05	-7668 to 1145

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

Capan-1 Cell Line in General Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	2527	1.553	P > 0.05	-1223 to 6276
1u vs B2-2	6778	3.762	P < 0.001	2623 to 10930

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	1219	0.6358	P > 0.05	-3188 to 5625
1u vs B2-2	8500	4.435	P < 0.001	4094 to 12910

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

Capan-1 CELL LINE

ETHANOL MEDIA CONDITIONS

Capan-1 Cell Line in Ethanol Media Conditions
ANOVA with Dunnett Post Test; 8 hr Data
Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	1423	0.9258	P > 0.05	-2995 to 5842
Cn vs 10p	631.0	0.3913	P > 0.05	-4003 to 5265
Cn vs 100p	2711	1.681	P > 0.05	-1923 to 7345
Cn vs 1n	1891	1.173	P > 0.05	-2744 to 6525
Cn vs 10n	672.8	0.3573	P > 0.05	-4739 to 6085
Cn vs 30n	3001	1.594	P > 0.05	-2411 to 8413
Cn vs 100n	3817	2.482	P > 0.05	-602.0 to 8235
Cn vs 1u	4243	2.760	P > 0.05	-175.8 to 8661
Cn vs B1-1	2762	1.713	P > 0.05	-1872 to 7397
Cn vs B1-2	943.2	0.5487	P > 0.05	-3997 to 5883
Cn vs B2-1	3729	2.425	P > 0.05	-689.6 to 8148
Cn vs B2-2	11430	7.437	P < 0.01	7016 to 15850

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10p	-792.4	0.9180	P > 0.05	-3256 to 1671
CnE vs 100p	1288	1.492	P > 0.05	-1176 to 3751
CnE vs 1n	467.4	0.5416	P > 0.05	-1996 to 2931
CnE vs 10n	-750.5	0.7446	P > 0.05	-3627 to 2126
CnE vs 30n	1578	1.565	P > 0.05	-1299 to 4454
CnE vs 100n	2393	2.908	P < 0.05	44.66 to 4742
CnE vs 1u	2820	3.426	P < 0.05	470.8 to 5168
CnE vs B1-1	1339	1.551	P > 0.05	-1124 to 3802
CnE vs B1-2	-480.2	0.5219	P > 0.05	-3106 to 2146
CnE vs B2-1	2306	2.802	P > 0.05	-43.01 to 4654
CnE vs B2-2	10010	12.16	P < 0.01	7662 to 12360

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

Capan-1 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 8 hr Data Ethanol Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cn vs CnE	192.2	0.2468	P > 0.05	-2090 to 2474
Cn vs 10p	2114	2.177	P > 0.05	-731.8 to 4960
Cn vs 100p	1125	1.445	P > 0.05	-1157 to 3407
Cn vs 1n	736.2	0.8683	P > 0.05	-1748 to 3220
Cn vs 10n	1835	2.164	P > 0.05	-649.3 to 4319
Cn vs 30n	1012	1.300	P > 0.05	-1269 to 3294
Cn vs 100n	4167	4.915	P < 0.01	1683 to 6651
Cn vs 1u	3820	4.906	P < 0.01	1539 to 6102
Cn vs B1-1	2189	2.582	P > 0.05	-295.3 to 4673
Cn vs B1-2	1345	1.586	P > 0.05	-1140 to 3829
Cn vs B2-1	1823	2.593	P > 0.05	-237.1 to 3882
Cn vs B2-2	10290	12.14	P < 0.01	7809 to 12780

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10p	1922	1.883	P > 0.05	-1058 to 4902
CnE vs 100p	933.0	1.120	P > 0.05	-1501 to 3367
CnE vs 1n	544.0	0.6043	P > 0.05	-2085 to 3173
CnE vs 10n	1643	1.825	P > 0.05	-985.8 to 4271
CnE vs 30n	820.3	0.9842	P > 0.05	-1613 to 3254
CnE vs 100n	3975	4.415	P < 0.01	1346 to 6603
CnE vs 1u	3628	4.354	P < 0.01	1195 to 6062
CnE vs B1-1	1997	2.218	P > 0.05	-631.8 to 4625
CnE vs B1-2	1152	1.280	P > 0.05	-1476 to 3781
CnE vs B2-1	1631	2.143	P > 0.05	-591.0 to 3852
CnE vs B2-2	10100	11.22	P < 0.01	7472 to 12730

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

Capan-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	51.40	0.03052	P > 0.05	-3834 to 3937
1u vs B1-2	-3300	1.920	P > 0.05	-7265 to 665.6

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	1064	1.200	P > 0.05	-1016 to 3143
1u vs B1-2	-2476	2.792	P < 0.05	-4555 to -396.6

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

Capan-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
8 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	1018	0.6313	P > 0.05	-2702 to 4738
1u vs B2-2	7192	4.678	P < 0.001	3645 to 10740

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	697.5	0.9308	P > 0.05	-1060 to 2455
1u vs B2-2	6473	7.300	P < 0.001	4393 to 8552

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

Panc-1 CELL LINE

GENERAL MEDIA CONDITIONS

Panc-1 Cell Line in General Media Conditions
ANOVA with Dunnett Post Test; 4 hr Data
Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	-423.8	0.3510	P > 0.05	-3862 to 3015
Cntl vs 100p	-1516	1.209	P > 0.05	-5090 to 2057
Cntl vs 1n	997.9	0.8266	P > 0.05	-2440 to 4436
Cntl vs 10n	456.3	0.3636	P > 0.05	-3117 to 4030
Cntl vs 30n	2137	1.703	P > 0.05	-1436 to 5710
Cntl vs 100n	1700	1.355	P > 0.05	-1873 to 5273
Cntl vs 1u	6758	5.387	P < 0.01	3185 to 10330
Cntl vs b1-1	594.4	0.4924	P > 0.05	-2844 to 4033
Cntl vs b1-2	4726	3.766	P < 0.01	1152 to 8299
Cntl vs b2-1	3598	2.980	P < 0.05	159.4 to 7036
Cntl vs b2-2	13980	11.15	P < 0.01	10410 to 17560

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs 10p	2111	2.498	P > 0.05	-294.5 to 4516
Cntl vs 100p	1128	1.284	P > 0.05	-1372 to 3628
Cntl vs 1n	2876	3.275	P < 0.05	376.4 to 5376
Cntl vs 10n	3549	4.041	P < 0.01	1049 to 6049
Cntl vs 30n	5644	6.426	P < 0.01	3145 to 8144
Cntl vs 100n	6303	7.175	P < 0.01	3803 to 8802
Cntl vs 1u	8372	9.531	P < 0.01	5872 to 10870
Cntl vs b1-1	3249	3.844	P < 0.01	843.8 to 5655
Cntl vs b1-2	2340	2.769	P > 0.05	-65.20 to 4746
Cntl vs b2-1	5126	6.065	P < 0.01	2720 to 7531
Cntl vs b2-2	12530	14.82	P < 0.01	10120 to 14930

Run 2

Legend:

Cntl=control with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1uM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1uM NNK

Panc-1 Cell Line in General Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
4 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b1-1	2111	1.864	P > 0.05	-504.9 to 4726
1u vs b1-2	-2033	1.718	P > 0.05	-4765 to 699.4

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b1-1	2121	2.676	P < 0.05	291.3 to 3951
1u vs b1-2	-6032	7.607	P < 0.001	-7862 to -4202

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

Panc-1 Cell Line in General Media Conditions ANOVA with Bonferroni Multiple Comparison Test; 4 hr Data NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b2-1	5114	4.516	P < 0.001	2498 to 7730
1u vs b2-2	7226	6.109	P < 0.001	4494 to 9958

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs b2-1	3998	5.043	P < 0.001	2168 to 5828
1u vs b2-2	4157	5.243	P < 0.001	2327 to 5987

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

Panc-1 CELL LINE

ETHANOL MEDIA CONDITIONS

Panc-1 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 4 hr Data Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs CnE	-2086	1.370	P > 0.05	-6455 to 2283
Cntl vs 10p	226.4	0.1431	P > 0.05	-4314 to 4767
Cntl vs 100p	1156	0.7308	P > 0.05	-3384 to 5696
Cntl vs 1n	941.9	0.6188	P > 0.05	-3427 to 5311
Cntl vs 10n	2865	1.811	P > 0.05	-1675 to 7405
Cntl vs 30n	3871	2.321	P > 0.05	-914.8 to 8657
Cntl vs 100n	5693	3.161	P < 0.05	523.6 to 10860
Cntl vs 1u	-2140	1.406	P > 0.05	-6508 to 2229
Cntl vs B1-1	-2104	1.382	P > 0.05	-6473 to 2264
Cntl vs B1-2	-2129	1.399	P > 0.05	-6498 to 2240
Cntl vs B2-1	-353.8	0.2324	P > 0.05	-4723 to 4015
Cntl vs B2-2	11880	7.805	P < 0.01	7513 to 16250

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Cntl vs CnE	-8657	5.824	P < 0.01	-12930 to -4382
Cntl vs 10p	-10910	6.203	P < 0.01	-15970 to -5851
Cntl vs 100p	-7702	4.986	P < 0.01	-12140 to -3259
Cntl vs 1n	-6231	4.192	P < 0.01	-10510 to -1956
Cntl vs 10n	53.45	0.03460	P > 0.05	-4389 to 4496
Cntl vs 30n	1108	0.7452	P > 0.05	-3167 to 5383
Cntl vs 100n	7205	4.425	P < 0.01	2521 to 11890
Cntl vs 1u	-177.3	0.1148	P > 0.05	-4620 to 4265
Cntl vs B1-1	4362	2.824	P > 0.05	-80.67 to 8805
Cntl vs B1-2	5554	3.736	P < 0.01	1279 to 9829
Cntl vs B2-1	6900	4.238	P < 0.01	2217 to 11580
Cntl vs B2-2	16510	11.11	P < 0.01	12240 to 20790

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

Panc-1 Cell Line in Ethanol Media Conditions ANOVA with Dunnett Post Test; 4 hr Data Ethanol Control vs. All Treatment Groups

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10p	2313	1.700	P > 0.05	-1559 to 6184
CnE vs 100p	3242	2.384	P > 0.05	-629.1 to 7113
CnE vs 1n	3028	2.335	P > 0.05	-663.1 to 6719
CnE vs 10n	4951	3.640	P < 0.01	1080 to 8822
CnE vs 30n	5957	4.108	P < 0.01	1830 to 10080
CnE vs 100n	7779	4.897	P < 0.01	3258 to 12300
CnE vs 1u	-53.50	0.04125	P > 0.05	-3745 to 3638
CnE vs B1-1	-18.33	0.01413	P > 0.05	-3709 to 3673
CnE vs B1-2	-42.83	0.03303	P > 0.05	-3734 to 3648
CnE vs B2-1	1732	1.336	P > 0.05	-1959 to 5423
CnE vs B2-2	13970	10.77	P < 0.01	10280 to 17660

Run 1

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
CnE vs 10p	-2252	1.463	P > 0.05	-6643 to 2139
CnE vs 100p	955.5	0.7248	P > 0.05	-2804 to 4715
CnE vs 1n	2426	1.930	P > 0.05	-1159 to 6011
CnE vs 10n	8711	6.607	P < 0.01	4951 to 12470
CnE vs 30n	9765	7.768	P < 0.01	6180 to 13350
CnE vs 100n	15860	11.29	P < 0.01	11850 to 19870
CnE vs 1u	8480	6.432	P < 0.01	4720 to 12240
CnE vs B1-1	13020	9.875	P < 0.01	9259 to 16780
CnE vs B1-2	14210	11.31	P < 0.01	10630 to 17800
CnE vs B2-1	15560	11.07	P < 0.01	11550 to 19570
CnE vs B2-2	25170	20.02	P < 0.01	21590 to 28760

Run 2

Legend:

Cntl=control in general media with PBS and no NNK
CnE=control in ethanol media with PBS and no NNK
b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM (β1 antagonist) + 1 μM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-1=10 nM ICI 118,551 (β2 antagonist) + 1 μM NNK

Panc-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
4 hr Data
NNK vs. β1 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	-3260	2.283	P > 0.05	-6551 to 30.21
1u vs B1-2	10.67	0.007835	P > 0.05	-3127 to 3148

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B1-1	12060	8.283	P < 0.001	8702 to 15430
1u vs B1-2	5731	4.110	P < 0.001	2513 to 8950

Run 2

Legend:

b1-1=10 nM atenolol (β1 antagonist) + 100 pM NNK
b1-2=100 μM atenolol (β1 antagonist) + 1 μM NNK

Panc-1 Cell Line in Ethanol Media Conditions
ANOVA with Bonferroni Multiple Comparison Test;
4 hr Data
NNK vs. β2 Antagonist

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	-1510	1.057	P > 0.05	-4800 to 1781
1u vs B2-2	14020	10.30	P < 0.001	10880 to 17160

Run 1

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
100p vs B2-1	14600	9.453	P < 0.001	11040 to 18170
1u vs B2-2	16690	11.97	P < 0.001	13470 to 19910

Run 2

Legend:

b2-1=10 nM ICI 118,551 (β2 antagonist) + 100 pM NNK
b2-2=100 μM ICI 118,551 (β2 antagonist) + 1 μM NNK

PART IV

SUMMARY

Chapter 4: Summary

Pancreatic carcinoma is a dismal disease with poor survival rates: 3-5%, 5 years; 9%, 2 years. Although it is the 4th leading cause of cancer death in people, it ranks 11th overall in cancer incidence (Gold, 1998; Kinjo, 1998). Tobacco and alcohol consumption are risk factors for a number of diseases which include pancreatic cancer (Driver, 1987; McCoy, 1979; Tugns, 1979; Park, 1995). The majority (75%) of pancreatic cancers are ductal adenocarcinoma. Ki-ras mutations and p53 mutations are very common in these cancers, 75% and 50% respectively. There are controversies in regard to the role risk factors and genetic mutations have in this cancer type.

Alcohol is known to cause pancreatitis. Chronic pancreatitis is believed to be a risk factor for pancreatic carcinoma. However, the causative link between these two factors is unclear (Gold, 1998; Gordis, 1993). Ki-ras mutations have been reported to lower in incidence in patients who smoke and consumed alcohol (Malats, 1997) In addition, it is reported that patients lacked Ki-ras and p53 mutations have decreased survival times (Dergham, 1997).

NNK, a by-product of nicotine nitrosation, is one of the most potent carcinogens in tobacco (Hecht, 1998). The role of NNK in development of lung cancer is well-documented. Using a transplacental hamster model of NNK induced carcinogenesis via intratracheal injection of NNK, it was noted that pretreatment with ethanol changed the target organ of cancer development from the respiratory tract to the pancreas (Schuller, 1993).

The research findings described in Parts II and III did not completely support the central hypothesis. The central hypothesis of this dissertation was that NNK would induce proliferation in selected pancreatic adenocarcinoma cell lines, would be blocked by subtype specific beta-adrenergic antagonists, and these effects would be enhanced by the presence of ethanol. However, the first specific hypotheses, which stated that ethanol treatment via the drinking water in pregnant hamsters would increase the receptor density of beta-adrenergic receptors in fetal pancreas and that pancreatic cell lines would contain beta-adrenergic receptors, were supported by radioligand binding studies and RT-PCR in Part I. The second specific hypotheses, that NNK would increase cell proliferation in these selected cell lines and that ethanol would enhance this effect, were examined by measuring DNA synthesis as a function tritiated thymidine incorporation in Part II. The findings did not support this hypothesis. The specific hypothesis, that the cell proliferation would by blocked with site selective antagonist, was partially supported.

Beta-adrenergic receptors were found to be present in fetal hamster pancreas and in 4 pancreatic carcinoma cell lines (BxPC-3, AsPC-1, Capan-1, Panc-1). Both β_1 and β_2 subtypes of adrenergic receptors were found to be present. Saturation binding studies conducted in the fetal hamster pancreas tissues revealed an increased number of beta-adrenergic receptors in the tissues derived from dams who had received ethanol compared to those dams who had received only water. Radioligand binding studies in these fetal tissues revealed that in the non-treated fetal pancreas, β_1 was the predominant subtype. Ethanol modulated the relative subtype proportions and changed the predominant subtype to β_2. Similar proportions of β_2 to β_1 found in the ethanol derived fetal pancreas were

present in the pancreatic carcinoma cell lines, particularly in the BxPC-3 and AsPC-1 cell lines. Although, three of these cell lines (BxPC-3, Capan-1, Panc-1) have been previously reported to possess beta-adrenergic receptors (Al-Nakkash, 1996), the relative proportions of specific beta-adrenergic subtypes has not been previously reported.

Although, the relative proportions of β_2 to β_1 receptors in the pancreatic cell lines were similar to those found in the ethanol derived fetal pancreas, it is not known what effect ethanol played in this finding. Risk factor histories from the patients in which the cell lines were derived were not available; therefore, it is not known to what extent these patients consumed alcohol. The majority of previous studies examining the effect of ethanol on density and affinity of beta-adrenergic receptors in a number of tissues have found a decrease in the overall numbers of receptors (Koga, 1993; Maki, 1990; Pohorecky, 1992). In general, the decrease has been attributed to down regulation of these receptors following increased levels of catecholamines resulting from ethanol exposure. These studies did not address differences in subtypes.

As demonstrated by the competition binding studies in the fetal pancreases, NNK bound to the beta-adrenergic receptors. An interesting finding was that ethanol treatment resulted in a shift in the binding curve to the left, suggesting an increased affinity for the receptors. Ethanol could have increased the affinity of NNK for the receptors or NNK could have been binding to increased numbers of β_2 receptors. It is not known whether NNK preferentially binds to one subtype. Competitive radioligand binding studies using site selective radioligands or studies using membranes derived from cells transfected with one subtype or the other could be used to more clearly determine this question.

In the transplacental hamster model in which dams pretreated with ethanol prior to receiving an intratracheal injection of NNK, the ethanol treatment modulated the target organ of NNK. Given those findings, it was not completely unexpected that NNK alone did not increase DNA synthesis in these selected pancreatic carcinoma cell lines. However, prior exposure to ethanol did not consistently result in or enhance the DNA synthesis. In some cases, there was inhibition compared to controls. This finding, too, was not consistent. The consistent finding in the cell proliferation assays was a statistically significant decrease in groups treated with the β_2 antagonist and NNK compared to controls and treatment groups containing equimolar concentrations of NNK alone. This finding, although not further enhanced, was consistent under ethanol media conditions. This finding suggest that there was inhibition of a mitogenic pathway driven by beta-adrenergic receptors. The finding suggest that this pathway could be primarily linked to β_2-adrenergic receptors. However, as stated in the discussion of Part III of this dissertation, this finding could be a reflection of 1 of 2 theories.

As eluded to in the aforementioned statement, β_1 and β_2-adrenergic receptors could operate through different signal transduction pathways in these cell lines. In general, both β_1 and β_2-adrenergic receptors activate adenylate cyclase through a G-protein and result in the formation of cAMP (Lefkowitz, 1990; Stiles, 1991). A β_2-adrenergic receptor coupled to cytosolic phospholipase A2, which triggers the release of arachidonic acid, has been identified in cardiomyocytes (Pavoine, 1999). It has recently been identified that cyclooxygenase-2 expression is up-regulated in human pancreatic adenocarcinomas (Tucker, 1999). These findings together suggest that there could be other mitogenic pathways in

pancreatic adenocarcinomas and could possibly be linked to β-adrenergic receptors, if not more specifically, β_2-adrenergic receptors. Questions as to whether a given signal transduction pathway was altered could be addressed by repeating similar studies and measuring for the presence or lack of specific second messengers involved in the pathways mentioned.

The other theory which might explain the inhibitory effect being observed in the presence of the β_2 antagonist is that β_2 receptors could be higher in number compared to β_1 receptors. This theory is supported by the findings in the competitive radioligand binding studies. However, ethanol treatment of these cell lines did not further enhance the findings. The radioligand studies in the fetal hamster pancreas suggest that ethanol could have enhanced this effect by increasing the proportion of β_2 receptors. However, these cell lines represent transformed carcinoma cells as opposed to "normal"/ non-diseased cells.

In addition to the above mentioned theories, it is possible that NNK and the β_2 antagonist could have interacted together to have resulted in the inhibition noted. This could be resolved by repeating the studies in the presence of the same concentrations of β_2 antagonist without NNK.

Pancreatic carcinoma cell lines were chosen to address the hypotheses in this dissertation due to the lack of a good normal human pancreatic ductal epithelial in vitro system. These cell lines represent transformed cells with maximized proliferative capabilities. Studies conducted in this laboratory have shown that NNK treatment activates MAP kinases in fetal hamster lung cells and small cell human lung cancer cell lines. This activation is far greater in the "normal" fetal cells than the cancer cell lines (Jull, 1999).

Revisiting an earlier topic in this discussion, the idea of a potential role for the arachidonic acid signal transduction pathway stimulated by β-adrenergic receptor activation in the development of pancreatic carcinoma is of increasing interest. The arachidonic acid pathway could be a link between chronic pancreatitis and the development of pancreatic carcinoma. Chronic pancreatitis is a feature of chronic alcohol consumption. Ethanol is cytotoxic; cytotoxic injury can result in cell death and secondary hyperplasia (Mufti, 1992). This effect has been linked to the potential effects of ethanol inducing precursor neoplastic lesions associated with chronic inflammatory conditions of the liver (Lieber, 1983; Mufti, 1992). Foci of hyperplasia and inflammation were observed in the offspring of female hamsters that received in utero ethanol (Schuller, 1993). As stated earlier in the summary discussion, it has recently been identified that cyclooxygenase -2 expression is up-regulated in human pancreatic adenocarcinomas (Tucker, 1999), and there is evidence in cardiomyocytes of a β_2-adrenergic receptor coupled to cytosolic phospholipase A2 which triggers the release of arachidonic acid (Pavoine, 1999).

The establishment of a role of the arachidonic acid pathway in pancreatic carcinogenesis coupled with the evidence of up-regulated expression of cyclooxygenase-2 (Tucker, 1999) suggests that there could be a potential therapeutic role of COX- 2 inhibitors. Likewise, if a role for β-adrenergic receptors is established, there may be some therapeutic potential. Obviously, given the fact that β-adrenergic receptors are present throughout the body, use of beta-blockers could have potential widespread systemic effects. However,

perhaps radio-chemotherapeutic or imaging agents could be linked to β-adrenergic antagonist and via a surgical procedure injected locally and increase the therapeutic /diagnostic armament available.

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VITA

Diann Weddle graduated Magna Cum Laude from North Carolina State University with a Bachelor of Science in Zoology in 1988. She received the Doctorate of Veterinary Medicine from the College of Veterinary Medicine at North Carolina State University in 1992. From 1992-1994, she was a National Research Service Award-NIH Postdoctoral Fellow in the department of Comparative Medicine at the Bowman Gray School of Medicine, Wake Forest University where she engaged in cardiovascular research in the area of atherosclerosis using the primate model. She was also an active participant in the ongoing medical and surgical care of colony nonhuman primates. From 1994-1998, she entered and completed an Anatomic and Surgical Pathology Residency in the Department of Pathology at the College of Veterinary Medicine, University of Tennessee in Knoxville. In 1999, she received her Doctor of Philosophy degree in Comparative and Experimental Medicine from the University of Tennessee from her work completed in the Experimental Oncology Laboratory in the Department of Pathology. She received the Dr. Dillman Award for Excellence in Anatomic and Clinical Pathology during her veterinary medical training. She is a member of the veterinary honor society, Phi Zeta. She holds medical licensure in North Carolina and is currently board-qualified in Veterinary Anatomic Pathology.